Helinski
Cohen
Clewell
Jackson
Hollaender

PLASMIDS IN BACTERIA

PLENUM

PLASMIDS IN BACTERIA

BASIC LIFE SCIENCES

Alexander Hollaender, General Editor

Council for Research Planning in Biological Sciences, Inc., Washington, D.C.

Recent volumes in the series:

Volume 18 TRENDS IN THE BIOLOGY OF FERMENTATIONS FOR FUELS AND CHEMICALS
Edited by Alexander Hollaender, Robert Rabson, Palmer Rogers, Anthony San Pietro, Raymond Valentine, and Ralph Wolfe

Volume 19 GENETIC ENGINEERING OF MICROORGANISMS FOR CHEMICALS
Edited by Alexander Hollaender, Ralph D. DeMoss, Samuel Kaplan, Jordan Konisky, Dwayne Savage, and Ralph S. Wolfe

Volume 20 MOLECULAR AND CELLULAR MECHANISMS OF MUTAGENESIS
Edited by J. F. Lemontt and W. M. Generoso

Volume 21 GENETIC TOXICOLOGY: An Agricultural Perspective
Edited by Raymond A. Fleck and Alexander Hollaender

Volume 22 PLANT CELL CULTURE IN CROP IMPROVEMENT
Edited by S. K. Sen and Kenneth L. Giles

Volume 23 INDUCED MUTAGENESIS: Molecular Mechanisms and Their Implications for Environmental Protection
Edited by Christopher W. Lawrence

Volume 24 ORGAN AND SPECIES SPECIFICITY IN CHEMICAL CARCINOGENESIS
Edited by Robert Langenbach, Stephen Nesnow, and Jerry M. Rice

Volume 25 BASIC BIOLOGY OF NEW DEVELOPMENTS IN BIOTECHNOLOGY
Edited by Alexander Hollaender, Allen I. Laskin, and Palmer Rogers

Volume 26 GENETIC ENGINEERING OF PLANTS: An Agricultural Perspective
Edited by Tsune Kosuge, Carole P. Meredith, and Alexander Hollaender

Volume 27 NEUTRONS IN BIOLOGY
Edited by Benno P. Schoenborn

Volume 28 GENETIC CONTROL OF ENVIRONMENTAL POLLUTANTS
Edited by Gilbert S. Omenn and Alexander Hollaender

Volume 29 SISTER CHROMATID EXCHANGES: 25 Years of Experimental Research
Part A: The Nature of SCEs
Part B: Genetic Toxicology and Human Studies
Edited by Raymond R. Tice and Alexander Hollaender

Volume 30 PLASMIDS IN BACTERIA
Edited by Donald R. Helinski, Stanley N. Cohen, Don B. Clewell, David A. Jackson, and Alexander Hollaender

Volume 31 GENETIC CONSEQUENCES OF NUCLEOTIDE POOL IMBALANCE
Edited by Frederick J. de Serres

A Continuation Order Plan is available for this series. A continuation order will bring delivery of each new volume immediately upon publication. Volumes are billed only upon actual shipment. For further information please contact the publisher.

PLASMIDS IN BACTERIA

Edited by

Donald R. Helinski
University of California, San Diego
La Jolla, California

Stanley N. Cohen
Stanford University
Stanford, California

Don B. Clewell
University of Michigan
Ann Arbor, Michigan

David A. Jackson
Genex Corporation
Gaithersburg, Maryland

Alexander Hollaender
Council for Research Planning
in Biological Sciences, Inc.
Washington, D.C.

with
Lowell Hager, Samuel Kaplan,
and Jordan Konisky

Claire M. Wilson, *Technical Editor*
Council for Research Planning
in Biological Sciences, Inc.
Washington, D.C.

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Main entry under title:

Plasmids in bacteria.

(Basic life sciences; v. 30)

"Proceedings of a conference on plasmids in bacteria, held May 13–18, 1984, at the University of Illinois, Urbana, Illinois"—T.p. verso.

Includes bibliographical references and index.

1. Plasmids—Congresses. 2. Bacteria—Congresses. I. Helinski, Donald R. II. Series.

QR76.6.P57 1985 589.9'087322 84-26397

ISBN 0-306-41901-7

Proceedings of a conference on Plasmids in Bacteria, held May 13–18, 1984, at the University of Illinois, Urbana, Illinois

©1985 Plenum Press, New York

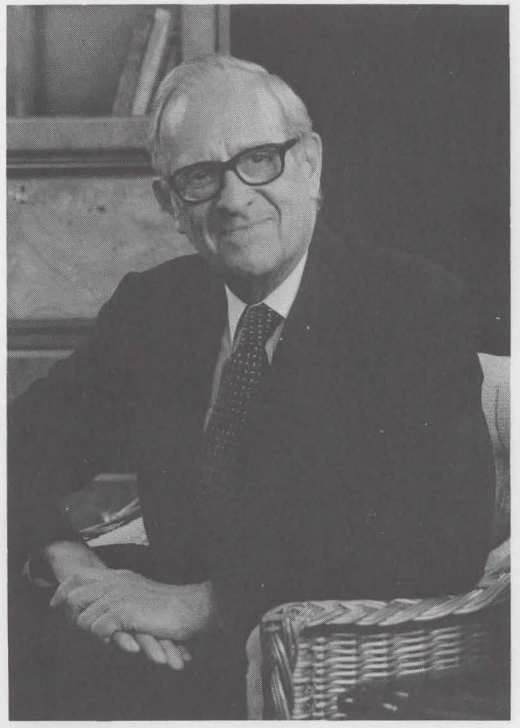
A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013

All rights reserved

No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording, or otherwise, without written permission from the Publisher

Printed in the United States of America

DEDICATION



The Editorial Committee of the conference on "Plasmids in Bacteria" dedicates this volume to Dr. I. C. Gunsalus in recognition and appreciation of his distinguished contributions to the fields of biochemistry and bacteriology. Gunny's independent scientific career spans some four decades and includes a number of innovative and fundamental contributions to our understanding of basic biochemical processes and bacterial metabolism. His early biochemical studies led to the isolation of the coenzymes pyridoxal phosphate and the discovery of lipoic acid. His later classical studies on the Pseudomonads provided the foundation for both fundamental and applied studies on the genetics and biochemistry of alkane and

terpene catabolic systems in bacteria. This pioneering research included the characterization of plasmids as carriers of the genes specifying the degradative enzymes responsible for these catabolic pathways. His illustrious career as a biochemist and bacteriologist has earned him a number of awards and honors. We add this dedication and extend our wishes for his continued success in the application of his extraordinary research ability to those research projects that currently command his interest.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous support of the National Science Foundation (Grant NSF PCM 84-06530), the National Cancer Institute (Grant 1 R13 CA 38747), the Argonne Universities Association Trust Fund, and the University of Illinois Research Board.

This material was prepared with the support of the U.S. Department of Energy (Grant DOE DEF G 2-84ER13201). However, any opinions, findings, conclusions, or recommendations expressed herein are those of the author(s) and do not necessarily reflect the views of DOE.

This work was also supported by the Department of Navy Grant N00014-84-G-0031 issued by the Office of Naval Research. The United States Government has a royalty-free license throughout the world in all copyrightable material contained herein.

Financial support was also derived from a number of industrial contributors as cited in alphabetical sequence: Abbott Laboratories; AMGen; Beckman; Biogen Corporation; CIBA-Geigy; Genex Corporation; International Biotechnologies, Inc.; Koppers Company, Incorporated; Eli Lilly; Merck, Sharp and Dohme Research Laboratories; Monsanto; New England Nuclear; Pfizer; Pioneer Hi-Bred; and UpJohn.

CONTENTS

Introduction 1
Stanley N. Cohen and Donald R. Helinski

STRUCTURE AND EVOLUTION

Plasmids as Organisms 3
Naomi Datta

Report on a Workshop: Structure and Function 17
Rüdiger Schmitt

Evolutionary Relevance of Genetic Rearrangements Involving Plasmids 21
Werner Arber, Christian Sengstag, Patrick Caspers, and Brian Dalrymple

Mechanisms of Transposition in Bacteria 33
Douglas E. Berg

Insertion of Transcriptional Elements Outside the Replication Region Can Interfere with Replication, Maintenance, and Stability of ColE1-Derived Plasmids 45
Hermann Bujard, Dietrich Stueber, Reiner Gentz, Ulrich Deuschle, and Ursula Peschke

Studies on the Transposition of IS1 53
Michael Chandler and David J. Galas

On the Transposition and Evolution of Tn1721 and its Relatives 79
Rüdiger Schmitt, Sabine Mötsch, Peter Rogowsky, Fernando de la Cruz, and John Grinsted

Repeated DNA Sequences Recombine 1,000 Times More Frequently in a Plasmid Than in the Chromosome of Bacillus Subtilis 93
L. Jannière, B. Niaudet, and S.D. Ehrlich

Mercury Resistance Transposon Tn813 Mediates Chromosome Transfer in Rhodopseudomonas sphaeroides and Intergeneric Transfer of pBR322 105
A. R. St. G. Bowen and J. M. Pemberton

Report on a Workshop: Plasmids in Unusual Systems 117
Simon Silver, John Pemberton, and Kenji Sakaguchi

REPLICATION, INCOMPATIBILITY, AND PARTITION

Chairman's Introduction: Replication, Incompatibility, and Partition 119
Kurt Nordström

Role of the π Initiation Protein and Direct Nucleotide Sequence Repeats in the Regulation of Plasmid R6K Replication 125
Marcin Filutowicz, Michael McEachern, Alan Greener, Pradip Mukhopadhyay, Elliott Uhlenhopp, Ross Durland, and Donald Helinski

Initiation of Replication of the Escherichia Coli Chromosomal Origin Reconstituted with Purified Enzymes 141
Jon M. Kaguni, LeRoy L. Bertsch, David Bramhill, James E. Flynn, Robert S. Fuller, Barbara Funnell, Satoko Maki, Tohru Ogawa, Kazuko Ogawa, Arie van der Ende, and Arthur Kornberg

Origin and Initiation Sites of λdv DNA Replication In Vitro .. 151
Toshiki Tsurimoto, Haruhiko Kouhara, and Kenichi Matsubara

Broad Host-Range Plasmid R1162: Replication, Incompatibility, and Copy-Number Control 173
Richard J. Meyer, Lung-Shen Lin, Kyunghoon Kim, and Michael A. Brasch

Control of Plasmid Replication: Theoretical Considerations and Practical Solutions 189
Kurt Nordström

The Partition Functions of P1, P7, and F Miniplasmids 215
Stuart Austin and Ann Abeles

Genetic Interactions of Broad Host-Range Plasmid RK2: Evidence for a Complex Replication Regulon 227
David H. Figurski, Calvin Young, Helen C. Schreiner, Robert F. Pohlman, David H. Bechhofer, Alice S. Prince, and Thomas F. D'Amico

Replication Determinants of the Broad Host Range Plasmid RSF1010 243
Peter Scholz, Volker Haring, Eberhard Scherzinger, Rudi Lurz, Miroslawa M. Bagdasarian, Heinz Schuster, and Michael Bagdasarian

Regulation of Replication and Maintenance Functions of Broad Host-Range Plasmid RK2 261
C. M. Thomas, C. A. Smith, V. Shingler, M. A. Cross, A. A. K. Hussain, and M. Pinkney

Control of Chromosome Replication in Bacteria 277
R. H. Pritchard

Stable Maintenance of Plasmid CLO DF13: Structural and Functional Relationships Between Replication Control, Partitioning, and Incompatibility 283
H. John J. Nijkamp, Bob van Gemen, Marcel J. J. Hakkaart, Arnold J. van Putten, and Eduard Veltkamp

Replication Control for PT181, An Indirectly Regulated Plasmid 299
Richard P. Novick, Steven J. Projan, C. Chandra Kumar, Stephen Carleton, Alexandra Gruss, Sarah K. Highlander, and John Kornblum

Construction of ColE1 RNA1 Mutants and Analysis of their Function In Vivo 321
Barry Polisky, Joseph Tamm, and Tim Fitzwater

Incompatibility and INCFII Plasmid Replication Control 335
Robert H. Rownd, David D. Womble, Xin-nian Dong, Verne A. Luckow, and Ru Ping Wu

P1 Plasmid Maintenance: A Paradigm of Precise Control 355
Dhruba K. Chattoraj, Ann L. Abeles, and Michael B. Yarmolinsky

Partitioning of the pSC101 Plasmid During Cell Division 383
Stanley N. Cohen, Christine A. Miller, William T. Tucker, Peter A. Meacock, and Peter Gustafsson

DNA-Protein Interaction at the Replication Origins of Plasmid Chromosomes 397
D. Bastia, C. Vocke, J. Germino, and J. Gray

Positive Regulation and Transcription Initiation of xyl Operons on TOL Plasmid 415
Teruko Nakazawa, Sachiye Inouye, and Atsushi Nakazawa

PLASMID TRANSFER

Chairmen's Introduction: Plasmid Transfer 431
Neil Willetts and Don B. Clewell

Linear Plasmids with Terminal Inverted Repeats Obtained from Streptomyces rochei and Kluyveromyces lactis 433
Kenji Sakaguchi, Hirohiko Hirochika, and Norio Gunge

Conjugal Plasmid Transfer in Bacillus Thuringiensis 453
John S. Chapman and Bruce C. Carlton

Mechanisms Essential for Stable Inheritance of Mini-F Plasmid 469
Sota Hiraga, Teru Ogura, Hirotada Mori, and Masafumi Tanaka

Sex Pheromones and Plasmid Transfer in Streptococcus Faecalis: A Pheromone, cAM373, Which is Also Excreted by Staphylococcus Aureus 489
Don B. Clewell, Florence Y. An, Bryan A. White, and Cynthia Gawron-Burke

General Genetic Recombination of Bacterial Plasmids 505
Amikam Cohen, Zipora Silberstein, Shimshon Broido, and Avraham Laban

The Origin of Plasmid DNA Transfer During Bacterial Conjugation 521
Donald G. Guiney, Gary Chikami, Cornelia Deiss, and Emanuel Yakobson

Genes and Gene Products Involved in the Synthesis of F-PILI 535
Susan Laine, Deanna Moore, Pushpa Kathir, and Karin Ippen-Ihler

Genetics of Clindamycin Resistance in Bacteroides 555
C. Jeffrey Smith, Melissa C. Liechty, Jeanette L. Rasmussen, and Francis L. Macrina

Effect of Chromosome Homology of Plasmid Transformation and Plasmid Conjugal Transfer in Haemophilus Influenzae 571
M. Balganesh and J.K. Setlow

Plasmid DNA Primases and Their Role in Bacterial Conjugation 585
B.M. Wilkins, L.K. Chatfield, C.C. Wymbs, and A. Merryweather

Promoters in the Transfer Region of Plasmid F 605
Phil Mullineaux and Neil Willetts

Conjugative Sex Plasmids of Streptomyces 615
D.A. Hopwood, D.J. Lydiate, F. Malpartida, and H.M. Wright

SPECIALIZED FUNCTIONS, STRUCTURE, AND EVOLUTION

The Structure and Source of Plasmid DNA Determine the Cloning Properties of Vectors for Bacillus Subtilis 635
P. M. Rabinovich, M. Ya. Haykinson, L. S. Arutyunova, Yu. V. Yomantas, and A. I. Stepanov

Shuttle Vector for Escherichia Coli, Pseudomonas Putida, and Pseudomonas Aeruginosa 657
A. A. Lushnikov, E. A. Andreevskaya, L. I. Lukashova, J. B. Lobach, and I. V. Domaradskij

Report on a Workshop: Plasmid Cloning Vehicles 663
J. Collins and C. Hershberger

Plasmids in the Degradation of Chlorinated Aromatic Compounds 667
D. Ghosal, I.-S. You, D.K. Chatterjee, and A.M. Chakrabarty

Notes on Metabolic Plasmid Organization and Expression 687
I.C. Gunsalus

The Structure of the mer Operon 707
Patricia Barrineau, Pearce Gilbert, W. James Jackson, Cheryl S. Jones, Anne O. Summers, and Sonya Wisdom

Analysis and Manipulation of Plasmid-Encoded Pathways for the Catabolism of Aromatic Compounds by Soil Bacteria 719
K.N. Timmis, P.R. Lehrbach, S. Harayama, R.H. Don, N. Mermod, S. Bas, R. Leppik, A.J. Weightman, W. Reineke, and H.J. Knackmuss

The Plasmid-Specified Aerobactin Iron Uptake System of Escherichia Coli 741
Peter H. Williams and Nicholas H. Carbonetti

Plasmid-Mediated Iron Sequestering Systems in Pathogenic Strains of Vibrio anguillarum and Escherichia Coli 759
J.H. Crosa, L.A. Actis, Y. Mitoma, J. Perez-Casal, M.E. Tolmasky, and M.A. Valvano

Chairman's Introduction: Specialized Functions 775
R.C. Clowes

Cloning of an Enzymatically Active Segment of the Exotoxin-A Gene of Pseudomonas Aeruginosa 777
Royston C. Clowes, Mark A. Mozola, Russell B. Wilson, Shin R. Hwang, and Rockford K. Draper

Structure, Function, and Regulation of the Plasmid-Encoded Hemolysin Determinant of Escherichia Coli 791
W. Goebel, J. Hacker, S. Knapp, I. Then, W. Wagner, C. Hughes, and A. Juarez

pIAA, A Virulence Plasmid in Pseudomonas Savastanoi 807
Tsune Kosuge, Curtis J. Palm, Steven V. Hutcheson, N. Louise, E. Glass, and Tetsuji Yamada

The Molecular Basis of Plant Cell Transformation by Agrobacterium Tumefaciens 815
E.W. Nester, R. Amasino, D. Akiyoshi, H. Klee, A. Montoya, and M.P. Gordon

Post-Transcriptional Regulation of Chloramphenicol Acetyl Transferase 823
Woo-Hyeon Byeon and Bernard Weisblum

Plasmid and Larval Toxin of Bacillus laterosporus 835
Yun-liu Fan and Wan-xue Xu

POSTERS
(Poster abstracts have been reproduced as submitted)

Replication, Incompatibility, Partition 841

Specialized Functions 881

Structure and Evolution 915

Transfer 937

Vectors 947

ROSTERS

Speakers 967

Participants 971

Index of Poster Authors 979

Index 983

INTRODUCTION

The study of bacterial plasmids has not always been as popular as it is today. For many years, the molecular biology of procaryotes was focused heavily on bacteriophage and plasmid investigations which were carried out in only a few laboratories. Whatever interest existed in plasmids concerned the role of these extrachromosomal elements in bacterial conjugation, genetic exchanges, and antibiotic resistance, as well as in the structure of plasmids themselves. Gradually, however, it became increasingly evident that many of the special characteristics displayed by bacteria of medical, agricultural, industrial, and environmental importance are determined by genes carried by plasmids, and this interest in plasmid-encoded functions, such as bacterial virulence properties (exotoxin production, serum resistance, adhesiveness), metabolism of organic compounds, plant tumor formation, and biological nitrogen fixation, led to increasing study of the plasmids that carry these genes. Investigations of other plasmid-related properties such as replication and recombination have yielded much information about fundamental biological processes; information having implications that extend far beyond the particular plasmids under study. Concurrently, plasmids were playing a key role in the discovery of bacterial transposable elements and were proving to be increasingly useful in the elucidation of mechanisms responsible for a variety of chromosomal rearrangement events in bacteria and plants. Their status as "minichromosomes" that could be isolated easily from bacterial cells and then reintroduced into other cells by transformation is of fundamental importance in this regard.

During the same time period, the major role of plasmids in the discovery and development of DNA cloning methodology and the importance of plasmids as vectors for the propagation and expression of a wide variety of foreign genes in bacteria was established and exploited with great success by basic and industrial scientists working in a number of biological and medical disciplines. As a consequence of these developments, interest in the biology of bacterial plasmids has broadened to the point where the results of research on plasmids are followed also by individuals who work primarily on the

biology of eucaryotes, and who view bacterial plasmids entirely as research tools. For plasmid biologists, DNA cloning techniques have provided a more detailed molecular understanding of the plasmids themselves, including their structure, replication, conjugal transfer, and inheritance. Thus, in a variety of ways, bacterial plasmids have played and continue to play a central role in the current revolution in biological research.

Significant recent progress in understanding the fundamental properties of plasmids, an explosive rise in the experimental use of plasmids as important tools in basic and applied studies, and the expanding list of specialized bacterial functions determined by plasmids provided the impetus for organizing this conference on "Plasmids in Bacteria." This is the first such international conference in the United States since the meeting held in Jackson, Wyoming in 1977. An international meeting of plasmids was held in Santa Domingo, Dominican Republic, in the early part of 1981.

It was the intention of the Organizing Committee to gather together outstanding workers in plasmids biology from throughout the world and to provide a forum to enable these scientists to present their work and interact with post-doctoral fellows and graduate students, as well as with other senior scientists. Whatever success has been achieved in facilitating the exchange of information and ideas has been due largely to the efforts of the Council for Research Planning in Biological Sciences, the faculty of the University of Illinois, and the staff of the Division of Conferences and Institutes of the University. Special gratitude is extended to Professors Sam Kaplan and Jordan Konisky of the University of Illinois, who have devoted an enormous amount of time and energy to the organization of this meeting. The enthusiasm and extensive scientific interactions that have occurred among the conference participants, as well as the high quality of the contributions to this volume, attest to the highly successful outcome of the efforts by the above individuals, and provide a sense of genuine satisfaction to all those who have played a role in the organization and conduct of this meeting.

Stanley N. Cohen
Donald R. Helinski

PLASMIDS AS ORGANISMS

Naomi Datta

Department of Bacteriology
Royal Postgraduate Medical School
Hammersmith Hospital
Du Cane Road
London W12 OHS, England

It is a quarter century since the publications of evidence for the transmissibility of drug resistance among Enterobacteria (1,48). Although it was not the beginning of plasmid research, it was that discovery that led to the great expansion of work on plasmids, their natural history, and the uses to which they can be put. By their practical importance, resistance or "R" plasmids forced themselves on the attention of microbiologists. The biochemical mechanisms of plasmid-determined resistance were investigated for their biological interest and because of the need to counteract their effects. Because drug-resistant clones can so easily be selected from large populations of bacteria, resistance genes are used to label DNA molecules, providing a practical basis for the genetic analysis of many prokaryotic and eukaryotic systems.

In 1959, no plasmid structure had been seen; the nature of plasmids was inferred from their genetic effects though, soon after that date, plasmid DNA was visualized in gradients (42) and conjugative pili were seen by electron microscopy (16). Close parallels between plasmids and viruses were recognized from the first, and "R factors" were classified, together with F and λ, as episomes, or genetic elements that could exist either autonomously or integrated into the chromosome of their host bacterium (34). Later the term episome, to denote one class of genetic element, was found to be more confusing than useful (30); plasmids do not necessarily integrate into

---

The title is adapted from F. M. Burnet, Virus as Organism (1945) Harvard University Press.

host chromosomes and their mode of integration differs from that of λ. Parallels between conjugative plasmids and bacteriophages are evident [Brinton (15) called the former, "epiviruses" or "sex viruses"]. However, plasmids without transfer genes cannot be excluded from a unifying concept. They, too, are extra genetic elements, transmissible by natural means and with characteristic host ranges among bacteria. The term plasmid as defined by Lederberg (38) includes nearly all of the organisms to be discussed in this volume, although possibly not conjugative transposons. I consider the latter, as well as orthodox plasmids, to be organisms according to the definition of Luria and co-authors (40), i.e., a unit of a continuous lineage with an individual evolutionary history.

Plasmids, such as R plasmids, that determine a specialized bacterial character, were at first considered to be analogous to F-primes, having acquired one or more bacterial genes by a process of insertion into and imprecise excision from the chromosome. We now know much more about the means by which plasmids acquire new genes; the discovery of the moveable genetic elements of prokaryotes, insertion sequences and transposons, that provide opportunities for recombination, both legitimate and illegitimate, and for other genetic rearrangements have, more than anything since 1959, changed our view of the natural history of plasmids.

PLASMID EPIDEMIOLOGY

Many studies of resistance plasmids have traced their spread through different bacteria either in hospitals or in the community (18,47,52,63). Where a plasmid remains unaltered during its spread, its identity can be established fairly easily by the pattern of resistance conferred and by its molecular weight. Quantitative or semiquantitative measurement of resistances can provide an accurate index of the particular resistance genes carried, especially if a number of related antibiotics are used in the tests. For example, the relative levels of resistance to various penicillins and cephalosporins determined by plasmids indicate which β-lactamase is present. Similarly, differential levels of resistance to a variety of aminoglycosides indicate which drug-modifying enzymes are present (56). The method used can be a a simple measurement of zones of inhibition around drug-impregnated discs, provided that test conditions are properly controlled (47). Interpretation is complicated if the plasmid determines gene products with overlapping effects, but the resistance patterns of 2 plasmids can still be compared and their identity established. Confirmation of identity is by showing that examples from different sources yield the same-sized fragments when digested with restriction enzymes. Often, however, variations from the original occur, usually DNA deletions or insertions. Some plasmids undergo such regular deletions, both in nature and in laboratory strains, that this behavior itself helps to identify them

for epidemiological purposes. For example, a plasmid (pHH1457) of about 110 megadaltons (Md), encoding multiple drug resistances, was found in isolates of Klebsiella aerogenes serotype K21 in an outbreak of hospital infection. It regularly underwent a deletion, losing about 25 Md of DNA, conjugative ability, and most of its resistance genes, though retaining clinically important genetamicin resistance. This deletion occurred in wild strains and in Rec+ and recA strains of Escherichia coli K12, at frequencies of about 0.25% per bacterial generation. The resistance pattern and this consistent behavior afforded recognition of the plasmid, whose Inc group was unknown. Its spread was traced to other hospital bacteria and, in the K21 Klebsiella, to several hospitals (12). Other examples of such consistent variations are known (18). Usually, however, plasmids undergo variation unpredictably and recognition of relationships between nonidentical plasmids requires some kind of classification.

Plasmids that inhabit a particular bacterial host can be divided into groups (Inc groups) by their incompatibility relationships. If each plasmid had a single DNA region encoding its determinants for replication and incompatibility, that region could be used to identify other plasmids of the same Inc group, either as a probe or as a miniplasmid or basic replicon with an inserted distinguishing marker in compatibility tests. But where replication and incompatibility functions have been analyzed they have been found to be controlled by complex genetic systems. We should not, then, expect that every naturally occurring plasmid will be classifiable into a discrete Inc group. However, many plasmids are so classifiable and DNA reassociation studies indicate that the Inc groups are respectable biological taxa.

Moreover, it is generally true that conjugative plasmids within an Inc group have identical or closely related transfer genes and their conjugative pili are morphologically and serologically similar, whereas plasmids of different groups have different conjugative genes and unrelated pili (13). There are exceptions. Take plasmids of groups IncFI and IncFII; they have closely related tra genes and pili, yet are compatible. This relationship could have resulted from recombination between 2 ancestral plasmids, let us say F itself (IncFI) and another (having the inc genes of IncFII, but without the F-like tra operon). Analysis shows that their lineage was less simple. More than one basic replicon is included in plasmids of IncFI and IncFII. Those of F (IncFI) and R100 (IncFII) are determined by sequences located at different sites (relative to their tra operons) (37,50). Yet recently, sequences have been found in F and in another IncFI plasmid, P307, homologous with a basic replicon of R100 and at the expected plasmid locus. Although they are there, the IncFII-like rep sequence of F seems to be nonfunctional (perhaps as the result of a γδ insertion) but it is functional in P307 (50,53). The way replication is controlled by F differs from that of the

IncFII R plasmids (43). These plasmids may well be recombinational assemblages of more than one replicon (24), but the evolutionary pathways cannot be traced on present data. Plasmids determining I pili, not only IncI but also IncB, IncK, and IncZ, belong to an analogous complex of groups. These plasmids all determine 2 kinds of pilus, thin and thick, serologically distinguishable and with different functions in conjugation (14). Relationships between the rep (vegetative replication) and inc genes of plasmids in these Inc groups have not been analyzed. Small serological differences among F-like pili or I-like pili are not related to the Inc groups within the F and I complexes.

The IncF and IncI complexes of conjugative plasmids are exceptional in that closely similar pili are determined by members of different Inc groups. Exceptions of the opposite kind--unrelated pili determined by plasmids of the same Inc group--have rarely been reported in nature. A plasmid of IncM, pDT201, specified F-like pili, quite different from typical M pili (60) and there are examples of naturally occurring cointegrations, giving plasmids with dual incompatibilities and expressing one or both sets of tra genes (46,60). Incompatibility grouping, although only an approximate criterion for relatedness of plasmids (53), has been successfully used in numerous studies of the epidemiology and natural history of plasmids (3,20,26).

"PREANTIBIOTIC" PLASMIDS OF ENTEROBACTERIACEAE

A collection of bacterial strains was made by E. D. G. Murray over the period 1917-1954, and when they were opened in 1980, over 400 separate isolates were viable. A high proportion (24%) of these possessed transfer genes, as demonstrated by their ability to mobilize small nonconjugative R plasmids in E. coli K12 (Tab. 1). Although the dates of collection were from 1917, some strains had been isolated earlier and were acquired by Murray from other collections; for example, one E. coli strain was isolated by Escherich about

Tab. 1. Murray Collection 1917-1954.

MURRAY COLLECTION 1917 - 1954

Genus	No. of strains	Colicin-ogenic	Tra^+ (mobilized tra^- R plasmid)
Salmonella	210	15	47
Shigella	140	31	35
Escherichia	32	10	8
Klebsiella	30	3	9
Proteus	16	0	3
Unidentified	5	0	2
Total	433	59	104 (24%)

Tab. 2. Plasmids from Murray Collection established in E. coli K12.

PLASMIDS FROM MURRAY COLLECTION ESTABLISHED IN E. coli K12

ColI	35
ColB	1
Tellurite-resistant	1
Cryptic	47
Total	84

1885. We do not know the dates and places of isolation of all the strains but we know they originated from many parts of the world including Europe, the Middle East, Northern Russia, India, and North and South America. From these strains, 84 conjugative plasmids were established in E. coli K12. None carried antibiotic-resistance, mercury-resistance, or haemolysin, 1 determined tellurite-resistance, and 36, colicinogeny (Tab. 2) (31). We classified the plasmids by incompatibility-testing with 25 R plasmids (21 belonging to known Inc groups and 4 odd, unclassified ones). We used the natural markers, col^+ or tellurite-resistance where possible, and labeled the cryptic plasmids with resistance transposons, converting them into R plasmids. The 35 ColI plasmids all belonged to IncI1. This result revealed likely epidemiological relationships. Clusters of $ColIa^+$ Shigella sonnei from 1937 and $ColIb^+$ Salmonella typhimurium from 1935 suggest that Murray collected multiple isolates from outbreaks in these years (Tab. 3). (Colicin-typing had not been introduced then, so he may not have been aware of any relationship.) If we count only 1 isolate from each of these clusters, the total number of plasmids in the study is reduced to 59 of which we could recognize or label 53. Of these, 40 (> 75%) belonged to Inc groups defined for R plasmids since 1970 (Tab. 4). Thus, these plasmids were common in enterobacteria and their acquisition of resistance genes came with the intense selective pressure exerted by the increasing use of antibiotics. It may be inferred that the resistance genes were acquired in a manner analogous to that in which we labeled the plasmids in the laboratory, as transposons (19).

Tab. 3. ColI plasmids from the Murray strains, all IncI1.

ColI PLASMIDS FROM THE MURRAY STRAINS, ALL IncI1

From:	Year	ColIa	ColIb
Shigella sonnei	1937	17	0
	1939	1	0
	1954	1	0
	?	1	0
Shigella flexneri	?	1	0
Salmonella typhimurium	1935	1	10
	1941	0	1
Salmonella spp.	1938	1	1

Tab. 4. Murray plasmids incompatibility results.

MURRAY PLASMIDS INCOMPATIBILITY RESULTS.	
Known groups	40
New groups	7
Unclassified	6
Total	53

The distribution of the plasmids classified was not exactly as expected for R plasmids today (Tab. 5). Plasmids of the F and I complexes and of IncN, which are common today, were represented, but plasmids of IncX were the most common. R plasmids of this group have been identified relatively infrequently but from diverse enterobacterial genera, from widely separate parts of the world and with different resistance genes (26,32,33). There was no epidemiological clustering of strains that yielded IncX plasmids. They were from 13 Salmonella strains of various serotypes, including S. typhi, collected at dates from 1917 to 1939, and 2 Klebsiella spp. dated 1932 and 1940.

Among the Murray plasmids there were none of Inc groups C, H, M, P, or W, all of which are common today as R plasmids. Their absence may be a simple sampling effect. We certainly do not assume that plasmids of these groups did not exist before the era of R plasmids. Some may have been common in bacterial genera other than those collected by Murray. Or they may have been relatively infrequent in enterobacteria and have spread widely only after acquiring resistance genes.

The 13 Murray plasmids that did not belong to any known group were tested for compatibility with one another, each against all.

Tab. 5. Classification of the Murray plasmids.

Classification of the Murray plasmids		
Inc group	No. of plasmids	Mol. wt (Md)
F II	3	35-50
F IV	4	35-50
I1	13	45-60
I2	1	40
B	2	50
N	2	25
X	15	30
Murray I	5	25-35
Murray II	2	?
Unclassified	6	30-110
Total	53	25-110

Two new Inc groups were revealed, containing 5 and 2 plasmids, apparently unrelated epidemiologially. Six, including the tellurite-R plasmid, were left unclassified.

No DNA reassociation study has been made to compare the Murray plasmids with present day R plasmids of the same Inc groups, but their close relationship seems probable. They had molecular weights of the order expected for their group (Tab. 5) and their conjugative pili were also as expected, recognized by acting as receptors for known pilus-specific phages and by immune electron microscopy (D. E. Bradley, pers. comm.). It was not only the conjugative pili of the Murray IncI1 and IncB plasmids that was as expected. Like known plasmids of the IncI complex, they determined, as part of their conjugative mechanism, the DNA primase that can substitute for host dnaG product (Ref. 14,64; D. E. Bradley, pers. comm.).

Our survey certainly underestimated the number of conjugative plasmids present in the Murray strains and made no study of nonconjugative ones. Plasmid profiles were not examined for all the original strains but DNA from 20 E. coli strains, negative in the mobilization test, was extracted (11) and run in gel electrophoresis. Plasmid bands were seen in 16 of these extracts, and in 8, migration distances indicated plasmids of over 30 Md, large enough to be conjugative. Moreover, electron microscopy (D.E. Bradley, pers. comm.) revealed the unexpected presence of constitutively produced F-like pili by a strain that had yielded an IncB plasmid. The latter, in E. coli K12, determined the pili, both thick and thin varieties, typical of IncB plasmids. The donor strain presumably harbored 2 unrelated conjugative plasmids, discovered accidentally since our protocol was not designed to isolate more than 1 transconjugant from each donor strain.

The conjugative plasmids in the Murray strains are not the only ones identified in "preantibiotic" cultures. Some were found in old cultures of Neisseria gonorrhoeae (51) and the prototype, F, was discovered in E. coli K12, isolated in 1922 (4).

STABILITY AND MUTABILITY

The plasmids in the Murray collection compared with present day R plasmids show that the functions designated inc and tra are conserved through many generations, vertical and horizontal. Specialized functions of plasmids are sometimes carried very stably through many generations, sometimes rapidly acquired or lost. Colicinogeny is an example of stable carriage. The ColI plasmids among the Murray strains closely resembled other early examples (59) as well as ColI plasmids (sometimes with additional drug resistance genes) found today. The association of one group of colicins (group II) with conjugative plasmids (colicins I with those of IncI, colicins V

or B with the IncF complex) and another group (group I) with small nonconjugative ones is well established (29). But the association is not immutable. Colicin I is determined by the IncFI, ColV+ plasmid ColVI-K94 and genes for colicins of group II have also been found on small nonconjugative plasmids (39). Antibiotic-resistance genes are those most evidently acquired and lost by plasmids within our working experience. Selection for drug resistance, especially in hospitals and on farms, has been intense, but it still seems almost incredible that resistance genes to so many unrelated antibiotics should have been accumulated in so short a time. For example, in London hospitals, now and for several years past, one can find bacteria harboring a plasmid, pTH1, of about 65 Md, of group IncM, with at least 9 resistance genes. Labigne-Roussel et al. (36) have demonstrated 4 steps by which these plasmids probably evolved from an ancestor, pIP135, identified in Paris in 1972. The current form has 4 more resistances and is about 20 Md larger than pIP135.

PLASMID LINEAGES

Plasmids can be considered as individual organisms and also as members of a large biological family divisible into smaller taxa. Results of research on the structure and function of some individual plasmids, such as ColE1, NR1 (or R100), R1, R6, RP4 (or RK2) throws light on the nature of plasmids generally and also demonstrates important differences between them.

It seems that some individual plasmids reside permanently in their host organisms. The history of bacteriology is short but that of bacterial disease is much longer. Should we believe that bubonic plague as described in the Bible (41) was caused by Yersinia pestis and, if so, was its pathogenicity plasmid-determined (8)? Was the dysentery that decimated armies dependent upon virulence plasmids in Shigella (35,54)? What is the lineage of the prophage that determines toxin-production in Corynebacterium diphtheriae (7)? There are no answers to such questions.

Probably the first subdivision of plasmids into taxa should be on the basis of host range. The plasmids of gram-positive bacteria have not been found in natural isolates of gram-negative ones, nor vice versa. Plasmids of gram-positive bacteria will replicate and be maintained in a variety of gram-positive hosts (23) and plasmids of gram-negative bacteria have various host ranges among gram-negative genera. [However, the replicative functions of Staphylococcal plasmids may be capable of expression in E. coli (5,27), and laboratory-constructed cointegrates of E. coli plasmids and Streptomyces plasmids will replicate in both hosts (55,61).]

It is often true that the G + C content of plasmid DNA resembles that of the host chromosome, indicating long-term residence (24,33). However, promiscuous plasmids such as RP4 or R300B can be

maintained in gram-negative genera with chromosomal DNA of very different G + C ratios. The base composition of plasmids could indicate their origins within bacterial genera or, equally, a tendency of what was originally foreign DNA to mutate to conform with that of the host cell.

The plasmid gene products that determine drug resistance also differ in gram-positive and gram-negative bacteria. This is somewhat surprising since gram-positive resistance genes, cloned in appropriate vectors, are expressed in E. coli (28,62). There must be opportunities in mixed populations of bacteria for miscegenation of genetic information, by means of transformation and rescue by transposition, yet the plasmid-determined genes such as encode penicillinases or chloramphenicol transacetylases are not found to cross the gram-positive/negative barrier (2,21,57). Miscegenation of this kind must be rare. Penicillinase was common in strains of Staphylococcus aureus by 1950 (6), although it was not until 1963 that its plasmid nature was recognized (44). In contrast, gonorrhea was treated with penicillin for 30 years before penicillinase plasmids appeared in Neisseria gonorrhoeae (49). This long delay illustrates lack of genetic exchange between gram-positive and gram-negative bacteria; the gonococcus had to wait for the world-wide dissemination of Tn3 in gram-negative bacteria before it had the opportunity of acquiring the TEM penicillinase. I do not mean to imply that the resistance genes evolved separately in gram-positive and gram-negative bacteria, only that in recent history interchange has been lacking. Common ancestors for the resistance genes seem almost certain given the similarities in amino acid sequences of penicillinases (2), chloramphenicol transacetylases (57) and aminoglycoside phosphotransferases (21,61) from gram-positive and gram-negative sources. The differences currently seen can hardly have developed in the last 40 years. One is led to conclude that clinically important resistance genes were established in gram-positive and gram-negative bacteria much longer ago than the human use of antibiotics, perhaps on plasmids, though not necessarily in the bacterial species known to man as pathogens.

I am not inclined to speculate on the ultimate origins of plasmids or related organisms. Small plasmids such as p15 must be among the simplest organisms in the world but whether they have contracted, discarding unnecessary complexities or alternatively, are in the first stages of evolution, I do not know.

CONCLUSION

Plasmids, like viruses, are organisms and so are some creatures that do not fit Lederberg's 1952 definition of plasmid; the conjugative transposons of gram-positive bacteria (25) and, in Haemophilus influenzae, the amplifiable sequences that can be identified as circularized copies (58). These have so many of the characteristics of

plasmids that they must be classed with them, yet do not replicate autonomously. In the enterobacteria, also, "plasmids" have been found without autonomous replication but they differ from conjugative transposons in behaving, at conjugation, like Hfrs (45), whereas the latter leave the host chromosome behind when they transfer themselves to a new host cell. Phages, plasmids and all transposing elements down to the smallest IS can logically be considered as one, unnamed, family of parasitic organisms with 2 modes of reproduction, one that results in vertical inheritance by the growing and dividing host and the other, an infective process that disseminates the organism horizontally, either intra- or intercellularly. I reject the hypothesis that transposition is nonreplicative (9), not on direct evidence but because I find it incredible. The unity of this unnamed family, then, is defined as an act of faith. Within it there is, obviously, great variation. Virulent phages, though not vertically inherited by their known hosts, cannot be classified separately. The degree of autonomy (independence, self-government) varies with the complexity of the organism but all have autonomy in their dissemination. I once considered R plasmids, and perhaps all plasmids, as bacterial symbionts, but although they may confer advantages on their host cells, I now see no need to assume that they must do so in order to survive. The advantages and disadvantages to a host cell of carrying extraneous DNA has been much debated (17,22)--an extra metabolic burden is disadvantageous, and special functions such as antibiotic-resistance may be advantageous. It has been shown that some transposons confer an unexplained survival advantage on their bacterial hosts in some circumstances (10). But if, as I believe, they all have the capacity for replicative dissemination, they are infective and will survive even if they make no direct return for the hospitality of their host cells.

Whether or not they confer advantages on individual host cells, they certainly favor bacterial populations in providing at each level: intergeneric, intercellular, and intracellular, the genetic flexibility necessary for adaptation to changing environments. Plasmids, as I have shown, can be stable organisms. Plasmids can also recombine, reassociate, rearrange, and be drastically altered. The same is probably true of their bacterial hosts of which we really know very little.

REFERENCES

1. Akiba, T. (1959) Mechanisms of resistance in Shigella. Proc. 15th General Meeting of the Japan Medical Association 5:229-305 [In Japanese, quoted by T. Watanabe (1963) Bact. Rev. 27:87-115].
2. Ambler, R.P. (1980) The structure of β-lactamases. Philos. Trans. R. Soc. Lond. (Biol.) 289-321-331.
3. Anderson, E.S., E.J. Threlfall, J.M. Carr, M.M. McConnell, and H.R. Smith (1977) Clonal distribution of resistance plasmid-carrying Salmonella typhimurium, mainly in Middle East. J. Hyg., (Camb.) 79:425-448.
4. Bachmann, B.J. (1972) Pedigrees of some mutant strains of Escherichia coli K12. Bact. Rev. 36:525-557.
5. Barany, F., J.D. Boeke, and A. Tomasz (1982) Staphylococcal plasmids that replicate and express erythromycin resistance in both Streptococcus pneumoniae and Escherichia coli. Proc. Natl. Acad. Sci., USA 79:2991-2995.
6. Barber, M., and J.E.M. Whitehead (1949) Bacteriophage types in penicillin-resistant staphylococcal infection. Brit. Med. J. 2:565.
7. Barksdale, L. (1970) Corynebacterium diphtheriae and its relatives. Bact. Rev. 34:378-422.
8. Ben-Gurion, R., and A. Shafferman (1981) Essential virulence determinants of different Yersinia species are carried on a common plasmid. Plasmid 5:183-187.
9. Berg, D.E., and C.M. Berg (1983) The prokaryotic transposable element Tn5. Biotechnology 1:417-435.
10. Biel, S.W., and D.L. Hartl (1983) Evolution of transposons: Natural selection for Tn5 in Escherichia coli K12. Genetics 103:581-592.
11. Birnboim, H.C., and J. Doly (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7:1513-1523.
12. Blenkharn, J.I., and V.M. Hughes (1982) Suction apparatus and hospital infection due to multiply-resistant Klebsiella aerogenes. J. Hosp. Infect. 3:173-178.
13. Bradley, D.E. (1980) Morphological and serological relationships of conjugative pili. Plasmid 4:155-169.
14. Bradley, D.E. (1984) Characteristics and function of thick and thin conjugative pili determined by transfer-repressed plasmids of incompatibility groups I1, I2, I5, B, K and Z. J. Gen. - Microb. 130:1489-1502
15. Brinton, C.C. (1971) The properties of sex pili the viral nature of "conjugal" genetic transfer systems, and some possible approaches to the control of bacterial drug resistance. Crit. Rev. Microbiol. 1:105-160.
16. Brinton, C.C., P. Gemski, and J. Carnanan (1964) A new type of bacterial pilus gentically controlled by the heredity factor of E. coli K12 and its role in chromosomal transfer. Proc. Natl. Acad. Sci., USA 52:776-783.
17. Campbell, A. (1981) Evolutionary significance of accessory DNA elements in bacteria. Ann. Rev. Microbiol. 35:55-83.
18. Chau, P.Y., J. Ling, E.J. Threlfall, and S.W.K. Im (1982) Genetic unstability of R plasmids in relation to the shift of drug resistance patterns in Salmonella johannesburg. J. Gen. Microbiol. 128:239-245.
19. Datta, N., and V.M. Hughes (1983) Plasmids of the same Inc groups in enterobacteria before and after the medical use of antibiotics. Nature 306:616-617.
20. Datta, N., V.M. Hughes, M.E. Nugent, and H. Richards (1979b)

Plasmids and transposons and their stability and mutability in bacteria isolated during an outbreak of hospital infection. Plasmid 2:182-196.

Davies, J., and G. Gray (1984) Evolutionary relationships among genes for antibiotic resistance. In Origins and Development of Adaptation, D. Evered and G.M. Collins, eds., pp. 219-228, CIBA Foundation Symposium 102, Pitman, London.

Doolittle, W.F., and C. Sapienza (1980) Selfish genes, the phenotype paradigm and genome evolution. Nature 284:601-603.

Engel, H.W.B., N. Soedirman, J.A. Rost, W.J. Leeuven, and J.D.A. van Embden (1980) Transferability of macrolide, lincomycin and streptogrammin resistance between group A, B and D streptococci, Streptococcus pneumoniae and Staphylococcus aureus. J. Bact. 142:407-413.

Falkow, S. (1975) Infectious Multiple Drug Resistance, Pion Ltd., London.

Franke, A.E., and D.B. Clewell (1981) Evidence for a chromosome-borne resistance transposon (Tn916) in Streptococcus faecalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bact. 145:494-502.

Frost, J.A., B. Rowe, J. Vandepitte, and E.J. Threlfall (1981) Plasmid characterization in the investigation of an epidemic caused by multiply resistant Shigella dysenteriae type 1 in Central Africa. Lancet 2:1074-1076.

Goze, A., and S.D. Ehrlich (1980) Replication of plasmids from Staphylococcus aureus in Escherichia coli. Proc. Natl. Acad. Sci., USA 77:7333-7337.

Hardy, K., and C. Haefeli (1982) Expression in Escherichia coli of a staphylococcal gene for resistance to MLS antibiotics. J. Bact. 152:524-526.

Hardy, K.G., G.G. Meynell, and J.E. Dowman (1973) Two major groups of colicine factors: Their evolutionary significance. Mol. Gen. Genet. 125:217-250.

Hayes, W. (1969) The Genetic of Bacteria and their Viruses 2nd edition, Blackwell, Oxford, pp.747-748.

Hughes, V.M., and N. Datta (1983) Conjugative plasmids in bacteria of the "pre-antibiotic" era. Nature 302:725-726.

Hughes, V.M., N. Datta, and M.C. Faiers (1981) Interhospital spread of a multiply resistant klebsiella. Brit. Med. J. 282:696.

Jacob, A.E., J.A. Shapiro, L. Yamamoto, D.I. Smith, S.N. Cohen, and D. Berg (1977) Plasmids studied in Escherichia coli and other enteric bacteria. In DNA Insertion Elements, Plasmids and Episomes, A.I. Bukhari, J.A. Shapiro, and S.L. Adhya, eds. Cold Spring Harbor Laboratory, USA, pp. 607-638.

Jacob, F., and E.L. Wollman (1958) Les épisomes, éléments génétiques ajoutés. C.R. Acad. Sci., Paris 247:154-156.

Kopecko, D.J., J. Holcombe, and S. Formal (1979) Molecular characterization of plasmics from virulent and spontaneously occurring avirulent colonial variants of Shigella flexneri. Infect. Immun. 24:580-582.

36. Labigne-Roussel, A., J. Witchitz, and P. Courvalin (1982) Evolution of disseminated Inc7-M plasmids encoding gentamicin resistance. Plasmid 8:215-231.

37. Lane, D., and R.C. Gardner (1979) Second EcoRI fragment of F capable of self-replication. J. Bact. 139:141-151.

38. Lederberg, J. (1952) Cell genetics and hereditory symbiosis. Physiol. Rev. 32:403-430.

39. Lehrbach, P.R., and P. Broda (1984) Molecular comparisons of plasmids isolated from colicinogenic strains of Escherichia coli. J. Gen. Microbiol. 130:401-410.

40. Luria, S.E., J.E. Darnell, D. Baltimore, and A. Campbell (1978) Gen. Virol., John Wiley and Sons, New York, p.4.

41. MacArthur, W.P. (1952) The plague of the Philistines (1 Sam. 5,6). Transactions of the Royal Society for Tropical Medicine and Hygiene, England, 46:209-212.

42. Marmur, J., R. Rownd, S. Falkow, L.S. Baron, C. Schildkraut, and P. Doty (1961) The nature of intergenetic episomal infection. Proc. Natl. Acad. Sci., USA 47:972-979.

43. Nordström, K. (1983) Review: Control of plasmid replication. Plasmid 9:1-7.

44. Novick, R.P. (1963) Analysis by transduction of mutations affecting penicillinase formation in Staphylococcus aureus. J. Gen. Microbiol. 33:121-136

45. Nugent, M.E. 1981) A conjugative 'plasmid' lacking autonomous replication. J. Gen. Microbiol. 126:305-310.

46. Nugent, M.E., K. Ellis, N. Datta, and D.E. Bradley (1982) pHH502, a plasmid with IncP and IncIα characters, loses the latter by a specific recA-independent deletion event. J. Gen. Microbiol. 128:2781-2790.

47. O'Brien, T.F., D.G. Ross, M.A. Guzman, A.A. Medeiros, R.W. Hedges, and D. Botstein (1980) Dissemination of an antibiotic resistance plasmid in hospital patient flora. Antimicrob. Ag. Chemother. 17:537-543.

48. Ochiai, K. (1959) Distribution and clinic of desentery caused by antibiotic-resistant strains. Proceedings 15th General Meeting of the Japan Medical Association 306-316 [In Japanese, quoted by T. Watanabe (1963) Bact. Rev. 27:87-115].

49. Phillips, I. (1976) β-lactamase-producing penicillin-resistant gonococcus. Lancet 2:656-657.

50. Picken, R.N., A.J. Naziatis, S. Saadi, and W.K. Maas (1984) Characterization of the basic replicons of the chimeric R/Ent plasmid pCG86 and the related Ent plasmid, P307. Plasmid.

51. Roberts, M., L. Elwell, and S. Falkow (1978) Introduction to the mechanisms of genetic exchange in the gonococcus: Plasmids and conjugation in Neisseria gonorrhoea. In Immunobiology of Neisseria gonorrhoea, G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young, eds. A.S.M., Washington, D.C.

52. Roberts, M., P. Piot, and S. Falkow (1979) The ecology of gonococcal plasmids. J. Gen. Microbiol. 114:491-494.

53. Saadi, S., W.K. Maas, and P.L. Bergquist (1984) RepFIC, a basic

replicon of IncFI plasmids that has homology with a basic replicon of IncFII plasmids. Plasmid.
54. Sansonetti, P.J., D.J. Kopecko, and S.B. Formal (1982) Involvement of a plasmid in the invasive ability of Schigella flexneri. Infect. Immun. 35:852-860.
55. Schottel, J.L., M.J. Bibb, and S.N. Cohen (1981) Cloning and expression in Streptomyces lividans of antibiotic resistance genes derived from Escherichia coli. J. Bact. 146:360-368.
56. Shannon, K., and I. Phillips (1982) Mechanisms of resistance to aminoglycosides in clinical isolates. J. Antimicrob. Chemother. 9:91-102.
57. Shaw, W.V. (1984) Bacterial resistance to chloramphenicol. Brit. Med. Bull. 40:36-41.
58. Spies, T. and R. Laufs (1983) Circularized copies of amplifiable resistance genes from Haemophilus influenzae plasmids. J. Bact. 156:1263-1267.
59. Stocker, B.A.D. (1966) Heterogeneity of I colicines and I colicine factors. Heredity 21:166-168.
60. Taylor, D.E., J.G. Levine, and D.E. Bradley (1981) In vivo formation of a plasmid cointegrate expressing two incompatibility phenotypes. Plasmid 5:233-244.
61. Thompson, C.J. and G.S. Gray (1983) Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids. Proc. Natl. Acad. Sci, USA 80:5190-5194.
62. Timmis, K., F. Cabello, and S.N. Cohen (1975) Cloning, isolation and characterisation of replication regions of complex plasmid genomes. Proc. Natl. Acad. Sci., USA 72:2242-2246.
63. Thompkins, L.S., J.J. Plorde, and S. Falkow (1980) Molecular analysis of R-factors from multiresistant nosocomial isolates. J. Infect. Dis. 141:625-636.
64. Willetts, N., and B. Wilkins (1984) Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. 48:24-41.

REPORT ON A WORKSHOP: STRUCTURE AND FUNCTION

Rüdiger Schmitt

Lehrstuhl für Genetik
Universität Regensburg
D-8400 Regensburg, Germany

Mobile genetic elements responsible for various genome rearrangements have been identified in prokaryotes and eukaryotes. They have been implicated in genome evolution, speciation, virus-induced cancers, and in the control of gene expression and thus were the focus of intense studies in recent years. These studies have reshaped our concepts of DNA. The storage of invariant genetic information suddenly turned into a dynamic structure, flexibly responding to adoptive needs and selective pressures. Due to their relative simplicity, bacterial transposons and IS elements have been used as paradigms for molecular analyses that revealed distinct features common to these elements: (i) their termini carry inverted repeats of 10-40 bp thought to serve as recognition sites for the transposition enzymes (transposases); (ii) the inverted repeats flank a central region containing genes or open reading frames that encode transposition functions; (iii) insertion into target replicons is accompanied by a short duplication of target DNA flanking the transposed element as direct repeats.

The central feature of mobile elements is their ability to integrate into foreign DNA sequences. This "illegitimate" recombination involving no apparent DNA homology is clearly distinguishable from the "legitimate" homologous recombination. The underlying mechanisms are still a matter of speculation, and it appears likely that different mechanisms apply to different elements and may even be operative in the same elements under changing conditions. At present, 2 alternatives are discussed, namely, conservative and replicative transposition. These are diagrammed in Fig. 1. Conservative transposition has been claimed for Tn5 (Berg) and Tn10 (Kleckner) to occur, whereas replication is involved in the transposition of Tn3 and its relatives. In the latter, cointegrates are

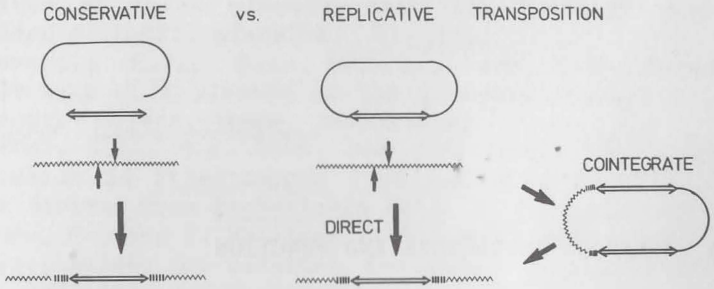


Fig. 1. Diagram of alternative mechanisms of transposition. Symbols: transposable element (⟺), donor (———), target (〰〰〰), direct repeats (▭), single-strand cuts (↑↓).

the preferred intermediates; they are resolved by a specific recombination event. Sometimes a bypass, termed "direct transposition," may be used instead. The duplication of target DNA flanking the transposed element is thought to be generated by the introduction of staggered nicks and subsequent DNA synthesis (Grindley & Sherratt). Replicative mechanisms are further subdivided into symmetric and asymmetric models depending on whether both ends of the element are nicked and ligated to the target DNA simultaneously (Shapiro; Arthur & Sherratt) or whether initiation occurs by nick-ligation at one end (Harshey & Bukhari; Galas & Chandler). There is supporting evidence for the latter to occur in phage Mu and in Tn1721, a relative of Tn3 (Schmitt).

Although transposable elements insert into various loci of a target replicon, most IS elements exhibit a preference for certain sites (Arber). Individual insertions into such "hot spots" do not necessarily involve the same target site, but rather center about distinct regions thought to contain a "recognition sequence" for transposase. This selection of target sites is reminiscent of mechanisms operative among class I restriction enzymes. In addition, AT-rich sequences (bearing resemblance to the termini of the element) are frequently found to flank the insertion.

IS elements and transposons are components of many plasmids. IS-mediated fusions, duplications, deletions, inversions, and the acquisition of new genes via transposons are still contributing to the rapid evolution of plasmids, that confer selective advantage to their hosts. On the other hand, little is known about the evolution of transposable elements themselves. An approach to this question relies on a comparison of members of a transposon family that have related sequences and interchangeable functions. Thus, Tn3- like

elements may have evolved in discrete steps involving the inverted ends, the transposition functions, and finally, different resistance determinants (Schmitt et al.). These elements have succeeded in crossing the barrier between gram negatives and gram positives as document by Tn917 that bears certain similarities to Tn3 (Shaw & Clewell).

Questions relating to host-element interactions and to the control of transposition frequencies are still unanswered. To the geneticist these elements thus open an exciting filed, with many challenges to the investigator, promising unprecedented insights into the structure and evolution of the genetic material.

EVOLUTIONARY RELEVANCE OF GENETIC REARRANGEMENTS INVOLVING PLASMIDS

Werner Arber, Christian Sengstag, Patrick Caspers,
and Brian Dalrymple

Department of Microbiology
Biozentrum of the University of Basel
Klingelbergstrasse 70, CH-4056
Basel, Switzerland

INTRODUCTION

Early molecular genetic studies with drug-resistance plasmids had indicated that transposable genetic elements, together with natural gene vectors, might play important roles in the biological evolution of microorganisms. More recently, these views have been widely confirmed. IS (insertion sequence) elements and transposons (1) have the characteristic ability to transpose to new locations on DNA, and often they also bring about additional genetic rearrangements, such as deletion, inversion, replicon fusion, and gene amplification (reviewed in Ref. 2). Alterations of gene functions, ranging from complete loss of an activity to improved gene expression, as well as gene fusion, can be the direct or indirect consequences of such genomic rearrangements. These, therefore, contribute to the vertical evolution of bacterial strains in which transposable elements reside. Via intermediary natural gene vectors, such as conjugative plasmids and viral genomes, and probably by still other means of horizontal gene flux (such as transformation and cell fusion), transposable elements can also make essential contributions to horizontal biological evolution. In this process, entire genes or functional domains of genes are acquired from other organisms and can provide a bacterium with new functional capacities (3).

Target selection criteria used by transposable elements are of great relevance for these evolutionary processes. Relatively little precise knowledge is available on these criteria, but it seems that they vary from one transposable element to another. Other relevant

questions concern the frequencies with which transpositional events and, subsequently, other genomic rearrangements occur, as well as their dependence on physiological and other environmental conditions. Here again, suggestive evidence is available that not all transposable elements follow the same rules.

The knowledge available on existence, structure, and functions of transposable elements comes largely from investigations with well-defined targets, mostly particular genes, operons, and small plasmids) on the one hand, and with particular, often selectable, transposable elements on the other. But, for obvious reasons, this approach could not answer to full satisfaction the questions raised above. Ideally, one would like to know of the existence of all transposable elements residing in a particular bacterial strain, to determine the frequency with which each of them mediates genetic rearrangements under various physiological conditions, and to understand the criteria applied in target selection. The importance of genomic rearrangements due to transposable elements could then be compared with that of other mechanisms of spontaneous mutagenesis.

For a number of technical reasons, it is practically impossible to carry out such investigations with the entire bacterial genome as target. Instead, large plasmids can offer a welcome opportunity to get at least partial answers to the questions raised. We would like to report here on the progress of relevant work carried out in recent years in our laboratory with the bacteriophage P1 genome, which is carried as a plasmid in the lysogenic condition in its host, Escherichia coli (E. coli) K12.

EXPERIMENTAL SYSTEM

Strategy for the Study of Spontaneous Prophage Mutants

The genome of the bacteriophage P1 and its relatives has a size of about 90 kb. A considerable part of the genetic information contained on the genome appears essential for vegetative phage reproduction, but not for maintenance of the lysogenic condition, for which relatively few genes are needed. In addition, the P1 DNA carries information for some functions unimportant for either vegetative reproduction or lysogeny, at least in E. coli K12 (4).

In the lysogenic condition, spontaneous mutations in most P1 genes can accumulate without affecting the prophage replication and maintenance. Many of these mutations may affect vegetative phage growth after induction of the lytic cycle. Others may alter the phenotype due to the expression of inessential functions, e.g., P1-specific restriction and modification. Still other alterations in the genetic information may remain silent under any experimental conditions applied.

Since many P1 genes are essential for the vegetative phage reproduction, an important fraction of the first class of mutations listed can, in principle, be considered as lethal. These mutations are easy to screen for by induction of lysogenic bacteria replicated or spotted onto a plate seeded with P1-indicator bacteria. This approach reveals the overall frequency of lethal spontaneous mutations of the P1 genome rather than of a particular gene. We consider this to be an interesting parameter, providing information on mutational activities affecting the phage as an individual organism.

Some nonlethal mutations can also be screened for, if they alter particular phenotypic traits. Most of such mutations, however, will fall into one or a few particular genes determining the phenotype considered. Such mutations will not be discussed further in this paper.

Frequency of Spontaneous Loss of the Ability of Phage P1 to Reproduce Vegetatively

In most of our experiments carried out so far, P1 lysogenic bacteria were kept for prolonged periods in rich growth medium either at room temperature or at 30°C. Every few days the cultures were diluted into fresh medium, each time allowing them to grow for 7 to 10 generations before again reaching the stationary phase. Screening for spontaneous P1 mutants after about 10 weeks, or 100 generations, revealed in the order of 10^{-3} to 10^{-2} mutant prophages per subclone tested.

Early experiments showed that roughly two-thirds of all P1 mutants isolated in this way were due to deletions. All studied deletions started at the single IS1 element resident in the wild-type P1 genome, and they extended to various sites on one or the other side of IS1 (5). We concluded that this IS1 element must be very deletogenic to the P1 genome, as had also been shown for IS1 elements at other locations (6). In later experiments we, therefore, used a close relative of P1, which is called P1-15 and does not carry any IS element in its genome (7). As expected, the overall frequency of spontaneous mutations in P1-15 is about 3 times lower than in P1, and the class of IS1-mediated deletion mutants is indeed missing.

One experiment was done with a P1-15 lysogenic strain, which was grown without interruption at 30°C in a turbidostat. Care was thereby taken to maintain the cell density constant, at 5×10^7 per ml. After a total of 200 generations spontaneous mutations affected about 10^{-4} prophages per subclone tested. This frequency is significantly lower than that found with the same strain grown with interruptions as described above in the same medium for 100 generations. This difference suggests a dependence of spontaneous mutagenesis on the physiology of the cell.

Most Spontaneous Prophage Mutants Are Due to Transposition of IS Elements

Several different mechanisms are expected to contribute to the spontaneous mutagenesis of the prophage (3). Since our main interest was directed towards transpositional events, the P1 plasmids of mutant prophages were extracted and analyzed for alterations in their size by restriction cleavage analysis. Care was taken that each individual mutant studied came from a different culture insuring its independent origin. Not taking into account the already described IS1-mediated deletion mutants of P1, this analysis showed that at least 90% of spontaneous prophage mutations carry an insertion or sequence duplication. Only a few mutants had no alteration in their genome size. Control isolates of nonmutated prophages usually had the wild-type genome size.

Further structural analysis and Southern hybridization experiments indicated that the increased genome size of the mutants is usually due to the transposition of a chromosomal IS element into the prophage. The most frequently found element is IS2 (28 of 44 studied independent mutants, Ref. 8). IS30 was found 3 times (9). Among the other insertions, IS1, IS5, and γδ were found, but not all insertions have yet been identified.

Study of the Target Sites of IS2 and IS30 Insertions

The locations of independently isolated insertions on the phage genome are strikingly nonrandom (Fig. 1). This applies particularly to IS2 and to IS30, which insert with high preference into the same distinct segment of the P1 genome. To investigate the target sites used by IS2 and by IS30, a 1756-bp segment covering the hot region for insertion of these elements was sequenced, as well as the junctions between some of the IS elements and their target (10). This study revealed that the target selection criteria used by IS2 and by IS30 must differ fundamentally.

Insertion sites of IS2. The insertion sites of 9 independent IS2-mediated mutations in the sequenced DNA segment appear to be more or less randomly distributed, in contrast to their distribution with regard to the entire phage genome (Fig. 1). The sequences at these 9 insertion sites show no common homology (10). This situation points to rather complex rules in target selection by IS2. Sengstag et al. (10) discussed 2 possible elements which they thought could contribute to attract IS2 preferentially into the hot region for insertion. These are, on the one hand, a segment of considerable, although imperfect, homology with an 85-bp long inside segment of IS2, and, on the other hand, the abundant occurrence of a pentanucleotide, GGTAT, in relative, although variable, proximity (less than 100 bp distant) to all studied IS2 insertions.

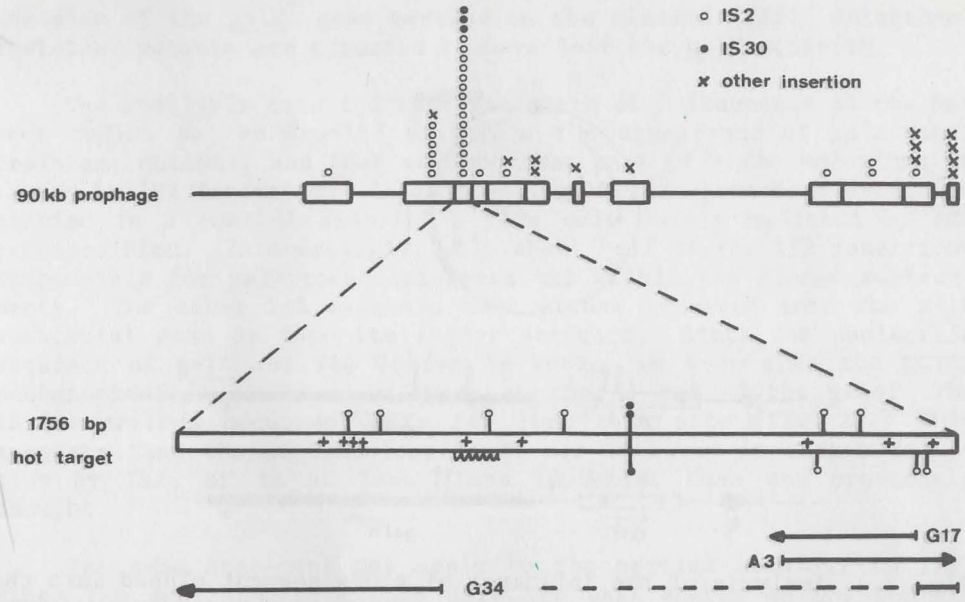


Fig. 1. Location of 44 independently isolated spontaneous insertion mutations on the phage P1 (or its close relative, P1-15) genome. In the upper map boxes represent the precision as obtained by restriction cleavage analysis. The locations of the 9 IS2 and the 3 IS30 insertions shown in the lower map were determined by nucleotide sequencing of the hot region for insertion. All 3 independent IS30-mediated mutations occurred at exactly the same target site. Symbols drawn above the map represent one orientation of the IS insertion, those below the map the other orientation. Data are taken from Ref. 8, which can be consulted for the reading frames in the 1756-bp segment. The wavy line indicates the location of a partial homology with an internal IS2 sequence. Crosses indicate the location of GGTAT sequences. The lines with arrow drawn below the map identify the cloned subfragments used in the experiments described in Fig. 2. The arrows point in the direction in which RNA synthesis proceeds in the constructions shown in Fig. 2.

We have started to investigate the possible importance of the homology to IS2, the pentanucleotide, and still other potential factors which could help to render the considered genome region a hot target for insertion. For this purpose, subfragments were cloned into a small plasmid which permits the selection of spontaneous mutations in the cloned segment or adjacent to it. The strategy used is outlined in Fig. 2 and it is based on the selection for galactose-resistant mutants among cells that are killed by the ex-

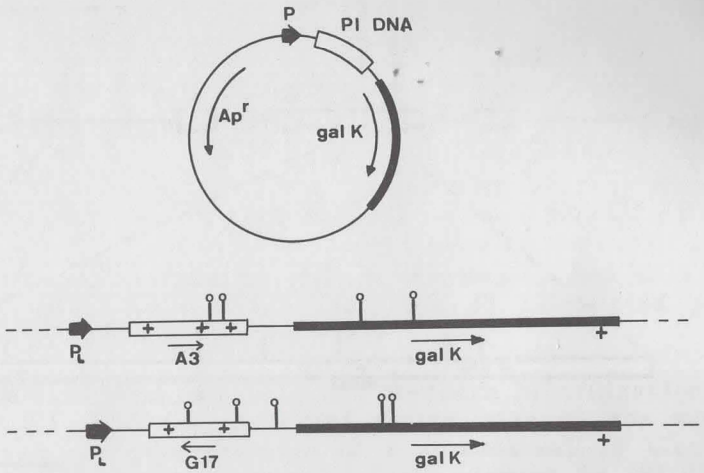


Fig. 2. Analysis of the influence of a DNA segment cloned into the leader sequence of galK on spontaneous, IS2-mediated mutagenesis. A selected DNA segment is cloned as schematically represented above into a small plasmid. The resulting plasmid is tested to see if expression of galK is still normal. A bacterial host deleted for the gal operon and grown in the presence of galactose is killed under conditions which induce the expression of galK in this plasmid. Rare galactose-resistant mutants are then selected and the proportion of mutations mediated by transpositional events is determined by restriction cleavage analysis of the plasmid. In the analysis shown in the lower part, the galK gene expression was under the control of the P_L promoter of phage λ. Among 5 galactose-resistant mutants of the plasmid carrying insert A3 (see Fig. 1), 4 had an increased genome size, in all cases due to the insertion of IS2. Similarly, 5 IS2 insertions and 4 mutations not affecting the plasmid size were analyzed in the plasmid carrying segment G17, which is a part of segment A3, but carried in the opposite orientation. The map positions of independent IS2 insertions are indicated by vertical lines with circles. Crosses indicate the location of GGTAT sequences. Another experiment with segment G34 (a fusion of the left part of the hot region for insertion with its extreme right part), cloned between the lacUV5 promoter and galK, gave similar results, while many fewer IS2 insertions were obtained with the plasmid not carrying any inserted Pl DNA. (Data are taken from C. Sengstag, Ph.D. thesis, University of Basel, 1984.)

pression of the galK$^+$ gene carried on the plasmid (11). Galactose-resistant mutants are expected to have lost the galK activity.

The available data indicate that both of 2 fragments of the hot spot region so far studied stimulate the appearance of galactose-resistant mutants, and that an important part of these mutations is caused by IS2 insertion. In contrast, galactose-resistant subclones studied in a control experiment were only rarely mediated by IS2 transposition. Interestingly, only about half of the IS2 insertions responsible for galactose-resistance map within the cloned subfragments. The other IS2 elements have either inserted into the galK structural gene or into its leader sequence. Since the nucleotide sequence of galK and its leader is known, we know that the GGTAT pentanucleotide occurs only once, at the 3' end of the gene. The IS2 insertions occurred quite far from this site (Fig. 2). This suggests that the pentanucleotide is not involved in target selection by IS2, or is at least less important than was previously thought.

The same statement may apply to the partial homology to IS2, since the subfragments cloned into the galK operon do not contain this part of the hot region for insertion. However, we have realized that the vector used to clone the subfragments also carried a 31-bp homology to IS2. As a matter of fact, this is a short internal sequence of IS2 (M. Rosenberg, pers. comm.), and it is split into a 19-bp and a 12-bp stretch upon in vitro insertion of the subfragments into the plasmid. Therefore, the potential role of homologies to IS2 remains uncertain. However, they cannot be the sole reason for the hot target property.

We expect to discover in further investigations, whether other particular structural elements in the cloned DNA segments may render the galK region a good target for IS2 insertion. If so, an influence at a distance might have to be assumed to explain the distribution of IS2 insertion sites as shown in Fig. 2. As a working hypothesis we assume that IS2 insertion may depend on the activation of the transposition process by a specific macromolecular interaction, e.g., of the transposase being bound to the ends of an IS2 element with a particular "activation" site on a potential target DNA, or vice versa. After activation, however, the actual site of insertion might then be selected in a more random process, at variable distances from the activation site. This hypothesis is based on the actual mechanism with which restriction endonuclease EcoK and its relatives function. They require an activation at a specific recognition site before they will cleave the same DNA molecule at a site chosen in each reaction at a variable distance from the activation site, often several thousand base pairs away (12).

Insertion site of IS30. In contrast to the situation with IS2, all 3 independently isolated mutations mediated by IS30 carry IS30

at exactly the same site of the P1 genome, and both orientations of IS30 were found. This site is in the hot region for IS2 insertion, but this may be a coincidence (Fig. 1).

Some years ago we had also found an IS30 by chance in an NR1 plasmid (5). This allowed us not only to delimit IS30, but also to determine that IS30 duplicates 2 bp of its insertion target (9). IS30 is 1221 bp long and has the characteristics of a typical IS element (13). Several E. coli K12 strains analyzed carry 2 to 4 copies of IS30 in the chromosome (5,9).

In Fig. 3, we compare the insertion site of IS30 in the P1 genome with the site at which IS30 is carried in the variant of NR1. One sees some striking homologies. However, while the integration site in P1 DNA is obviously a preferred insertion target, nothing is known about the frequency with which the target in NR1 is used. The latter could perhaps be a secondary target site. As do other IS elements, IS30 appears also to mediate deletion formation. A deletion which occurred in the NR1 variant was analyzed and its new junction with IS30 is also shown in Fig. 3. It reveals additional homology to the preferred target site in P1 DNA (9).

A few other targets for IS30 transposition were recently identified on the P1-15 genome. They served for IS30-mediated cointegration of a small plasmid carrying IS30 with the P1-15 genome, without abolishing the ability of P1-15 to reproduce vegetatively (9). We expect that sequencing of these sites might help to further elucidate the target specificity of IS30.

DISCUSSION

The prophage P1 plasmid represents the entire genome of a large bacteriophage with a relatively complex genetic setup. Most of

....TCATAAGTGCCAT ←1221 bp IS30→ ATCTCCTTTCTTA.... (IN P1 DNA)

....GCGAAATGCCAGC ←1221 bp IS30→ GCGTCGAATTGCG.... (IN NR1)

DELETION IN NR1: 1221 bp IS30→AGTACCTTTATTA....

Fig. 3. Insertion sites of IS30 in P1 DNA (see Fig. 1) and in NR1. To facilitate comparison, homologies between the target sites in P1 DNA (first line) and NR1 (second line) are written in larger letters. The 2 bp duplicated upon transposition are underlined. The third line gives the sequence adjacent to an IS30-mediated deletion in the NR1 variant. Here, larger letters identify those nucleotides homologous with the corresponding positions in the IS30 target of P1 DNA. (Data extracted from Ref. 9.)

these phage functions are essential for vegetative phage reproduction, but not for prophage propagation. The P1 prophage thus offers an ideal system for the study of spontaneous mutations affecting phage functions. This strategy is well known to bacteriophage geneticists (14,15). Although such mutations may in principle often be lethal, the lethality mainly affects phage reproduction. Therefore, mutations can be accumulated over the course of time in the lysogenic condition, and phenotypic manifestation can be studied at any chosen time by the induction of phage production. Subsequent analysis of the mutational alteration on the phage genome can reveal the mechanism underlying mutagenesis.

In the experiments reported here, transposition of IS elements obviously played the major role in spontaneous mutagenesis. Although IS2 was the most active in the production of spontaneous mutations, several other IS elements residing in the host chromosome also contributed to the production of mutations. The sum of these transpositional activities must be considered as more important for spontaneous mutagenesis of the phage genome as a whole than any other mechanism of mutagenesis. The study indeed allows one to directly compare frequencies of the different events affecting the functioning of the entire genome.

The analysis of transpositional mutations mediated by IS2 and by IS30 clearly revealed that these types of mutations do not occur at random on the P1 genome. Therefore, some genes will undergo spontaneous mutation much more frequently than others. One may therefore expect that some particular genes may represent bad targets for transposition and that these genes will not only show a lower rate of spontaneous mutagenesis, but that their spontaneous mutations may rather be caused by mechanisms other than transposition.

It has been known for some time that target selection criteria vary from one IS element to another (see Ref. 2). Some IS elements appear to select their target by specific recognition of a particular nucleotide sequence. IS30 may belong to this class. Other IS elements appear to proceed by a more complex mechanism, and IS2 may represent a prototype of this class. Although the distribution of their transposition targets in a short DNA segment may appear random, this does not necessarily hold for long DNA molecules, such as entire genomes. A regional specificity for transposition, together with the possibility of choosing a large number of particular sites for insertion, may be of great evolutionary relevance. Indeed, genetic rearrangements other than simple transposition may often also depend on the same criteria of target selection. And at least some of these events play important roles in gene fusion and operon fusion, which can give rise to new biological functions and to altered rules for the expression of existing genes. These mechanisms are of relevance in vertical as well as in horizontal evolution.

It is generally admitted that plasmids are excellent breeding grounds for new biological functions, particularly since their genetic content is often inessential for the host bacterium. This last point may be of relevance in attempts to generalize the conclusions made in this paper. One may expect that chromosomal genes, including the essential ones, may be used as transposition targets according to the same rules as discussed for phage genes. Such mutations might often also be lethal and thus not be propagated. However, there is good experimental evidence that transposition often occurs also at functionally inessential sites, both in bacterial chromosomes as well as in bacteriophage genomes. We, therefore, think that the transpositional mechanisms discussed here can be considered to make an important general contribution to the development of altered and new functions in the wide gene pool of microorganisms.

ACKNOWLEDGMENT

The continued support of our research by the Swiss National Science Foundation is acknowledged.

REFERENCES

1. Campbell, A., D. Berg, D. Bottstein, E. Lederberg, R. Novick, P. Starlinger, and W. Szybalski (1977) Nomenclature of transposable elements in prokaryotes. In DNA Insertion Elements, Plasmids, and Episomes, A.I. Bukhari, J.A. Shapiro, and S.L. Adhya, eds. Cold Spring Harbor Laboratory, pp. 15-22.
2. Iida, S., J. Meyer, and W. Arber (1983) Prokaryotic IS elements. In Mobile Genetic Elements, J.A. Shapiro, ed. Academic Press, New York, pp. 159-221.
3. Arber, W. (1984) Natural mechanisms of microbial evolution. In Genetic Manipulation: Impact on Man and Society, W. Arber, K. Illmensee, W.J. Peacock, and P. Starlinger, eds. The ICSU Press and Cambridge University Press, Cambridge, pp. 1-14.
4. Yarmolinsky, M. (1982) Bacteriophage P1. In Genetic Maps, Vol. 2. S.J. O'Brien ed., Natl. Cancer Inst., Frederick, Maryland, pp. 34-43.
5. Arber, W., S. Iida, H. Jütte, P. Caspers, J. Meyer, and C. Hänni (1978) Rearrangements of genetic material in Escherichia coli as observed on the bacteriophage P1 plasmid. Cold Spring Harbor Symp. Quant. Biol. 43:1197-1208.
6. Reif, H.J., and H. Saedler (1975) IS1 is involved in deletion formation in the gal region of E. coli K12. Mol. Gen. Genet. 137:17-28.
7. Meyer, J., S. Iida, and W. Arber (1983) Physical analysis of the genomes of hybrid phages between phage P1 and plasmid p15B. J. Mol. Biol. 165:191-195.

8. Sengstag, C., and W. Arber (1983) IS2 insertion is a major cause of spontaneous mutagenesis of the bacteriophage P1: Non-random distribution of target sites. EMBO 2:67-71.
9. Caspers, P., B. Dalrymple, S. Iida, and W. Arber (1984) IS30, a new insertion sequence of Escherichia coli K12. Mol. Gen. Genet. 196:68-73.
10. Sengstag, C., J.C.W. Shepherd, and W. Arber (1983) The sequence of the bacteriophage P1 genome region serving as hot target for IS2 insertion. EMBO 2:1777-1781.
11. Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
12. Yuan, R. (1981) Structure and mechanism of multifunctional restriction endonucleases. Ann. Rev. Biochem. 50:285-315.
13. Dalrymple, B., P. Caspers, and W. Arber (1984) Nucleotide sequence of the prokaryotic mobile genetic element IS30. EMBO (in press).
14. Jacob, F., and E.L. Wollman (1956) Recherches sur les bactéries lysogènes défectives. I. Déterminisme génétique de la morphogenèse chez un bactériophage tempéré. Ann. Inst. Pasteur 90:282-302.
15. Arber, W., and G. Kellenberger (1958) Study of the property of seven defective-lysogenic strains derived from Escherichia coli K12. Virology 5:458-475.

MECHANISMS OF TRANSPOSITION IN BACTERIA

Douglas E. Berg

Departments of Microbiology and Immunology and of Genetics
Washington University Medical School
St. Louis, Missouri 63110

ABSTRACT

The transposable elements of bacteria are diverse in size, functional arrangement, DNA sequence, and in their modes of transposition. We review here data suggesting that the kanamycin-resistance transposon Tn*5* moves without replicating (conservative transposition), but the ampicillin-resistance transposon Tn*3* is duplicated when it transposes, and that both the chloramphenicol-resistance transposon Tn*9* and bacteriophage Mu are replicated in some events but not in others. A model is presented in which conservative and replicative transposition are alternative branches of a single pathway.

INTRODUCTION

Transposable elements are DNA segments which move to new genomic sites by pathways distinct from those leading to homologous recombination. These elements were discovered by McClintock in the 1940s as the agents responsible for the intriguing variability of a class of unstable alleles in maize, and have subsequently been found in all groups of organisms. Transposable elements cause spontaneous mutations and genome rearrangements, and alter or modulate the expression of nearby immobile genes. Studies in bacteria have shown that transposition is independent of the recombination (*rec*) genes and of DNA sequence homology, and is mediated by element-specific proteins termed transposases which probably bind short DNA sequences repeated at each end of the element. Transposition in bacteria facilitates the flow of genes between distantly related species and transposable elements have become valuable laboratory tools, espe-

cially for work with bacterial species which had not been extensively analyzed using classical genetic approaches (for reviews, see Ref. 5,21).

There has been a great surge of interest in transposable elements during the last decade, much of it focused on the mechanism and regulation of transposition. Here we review studies of several groups of bacterial elements, and models of how they may transpose. The elements discussed are: (i) the kanamycin-resistance transposon Tn5 and its component IS50 elements, (ii) the chloramphenicol-resistance transposon Tn9 and its component IS1 elements, (iii) the ampicillin-resistance transposon Tn3, and (iv) bacteriophage Mu. Each of these elements encodes its own transposase and duplicates a short segment of target DNA when it inserts. The evidence suggests that IS50 and Tn5 transpose by a conservative (nonreplicative) mechanism, that Tn3 transposes by a replicative mechanism, and that Mu, IS1, and Tn9 transpose by both mechanisms.

MODELS OF TRANSPOSITION

Three general classes of transposition models have been advanced. The first and simplest, conservative transposition (Fig. 1), was developed to account for Tn5 transposition (3,4). It involves double-strand breaks at each end of the element, accompanied by staggered cuts (9 bp apart for Tn5) in the complementary strands of target DNA. The protruding target strands are joined to the element, and transposition is completed by repair synthesis which fills in the gaps and generates the 9-bp target sequence duplication which brackets Tn5. The ends of the vector DNA are not rejoined, and may serve as sites of exonucleolytic attack leading to loss of the vector. Because of the sensitive way in which replicon copy numbers are maintained, this loss would generally be compensated by the premature replication of a sibling of the vector molecule, one which had not participated directly in transposition and thus still retained an intact copy of the mobile element. This model accounts for the transposition of Tn5 and of its component IS element, IS50.

The second model of transposition, the bidirectional replicative model (Fig. 2) was developed to account for results obtained with Tn3 and with prophage Mu (2,20). In this model one DNA strand at each end of the element is cut and joined to staggered ends in the target DNA, forming a pair of primers for the replication of the element. The product, called a cointegrate, consists of vector and target replicons joined by directly repeated copies of the element. These cointegrates are unstable because they can be broken down (resolved) at a low rate by homologous recombination. In addition, Tn3 encodes a protein termed resolvase which breaks down cointegrates rapidly by a site-specific recombination reaction (12).

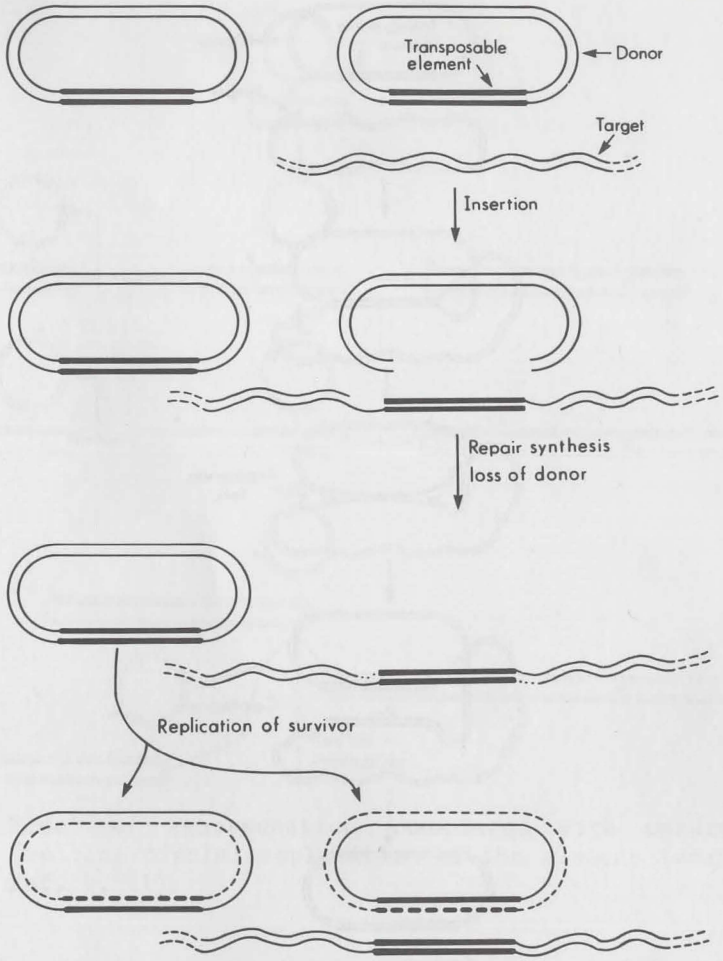


Fig. 1. Model of conservative transposition (adapted from Ref. 3-5).

The third model of transposition, the rolling circle model (Fig. 3), is based on the premise that all transposition is replicative, and was developed to account for results obtained with IS1 and with phage Mu (9,11). Transposition is initiated by the joining of one strand at just one end of the element to the target. Replication from the resulting fork proceeds unidirectionally and spins off a DNA tail consisting of alternating element and vector sequences. Termination occurs at a transposase recognition site which is opposite in orientation to that used to initiate transposition. No specific resolvase is required: either separate donor and target molecules (Fig. 3, left) or cointegrates (Fig. 3, right) result, depending on the way in which DNA strands are joined during termination.

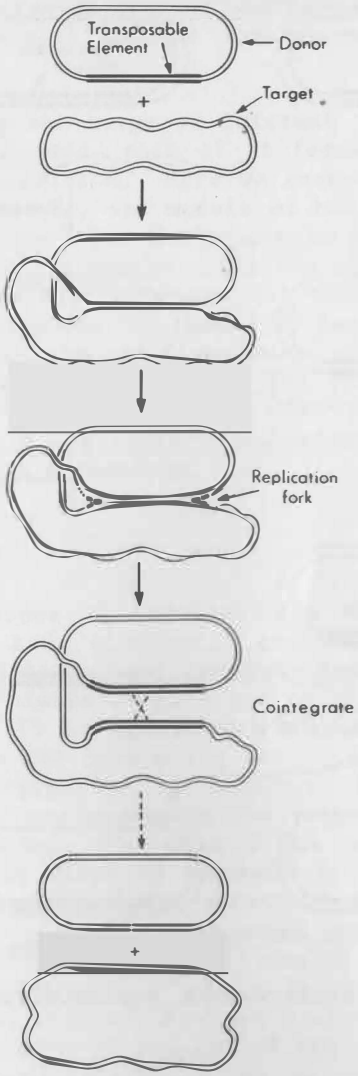


Fig. 2. Model of transposition associated with bidirectional replication of the mobile element (adapted from Ref. 2, 20). The final reaction, resolution, is indicated by a dashed arrow, since this reaction is separate from transposition, and for some elements occurs only by homologous recombination.

RESULTS BEARING ON TRANSPOSITION MODELS

Tn5: Conservative Transposition

The transposition of Tn5 in recA⁻ E. coli generates simple insertions of fragments of the donor molecule, not cointegrates con-

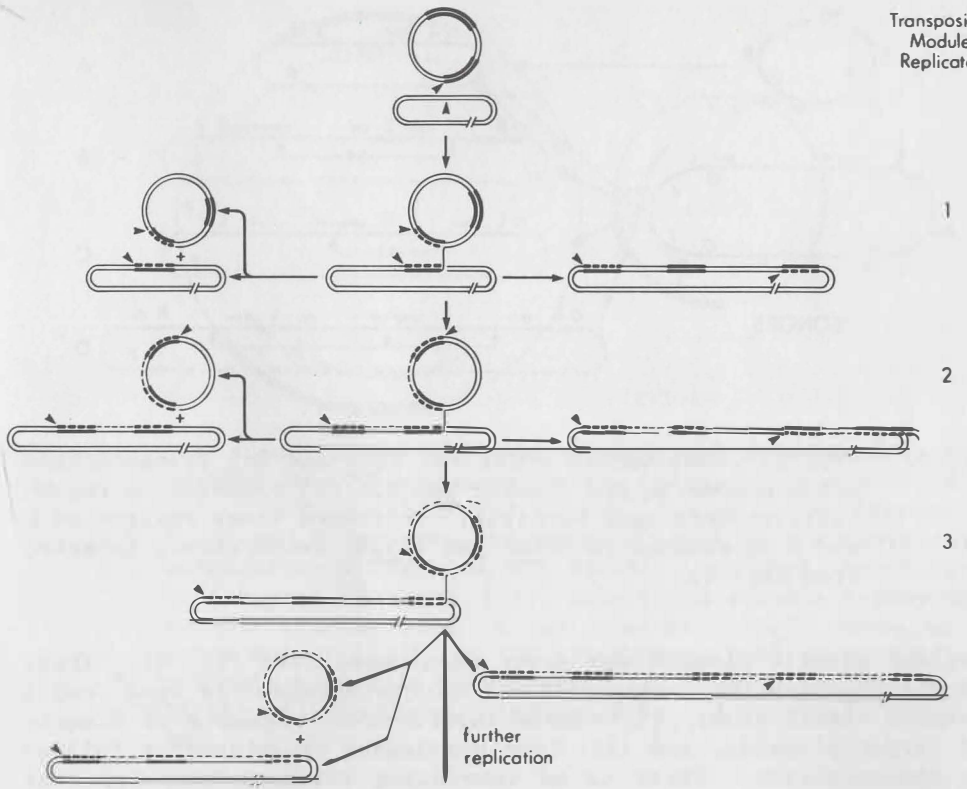


Fig. 3. Model of transposition associated with unidirectional (rolling circle) replication of the element (adapted from Ref. 9, 11).

taining the entire vector molecule, the target molecule, and duplicate copies of the element. For example, transposition of the kan determinant of Tn5 from monomeric pBR322::Tn5 plasmids is not associated with transposition of the vector amp or tet markers. In contrast, transposition of Tn5 from pBR322::Tn5 plasmids in recombination-proficient (recA⁺) cells is often associated with the co-transposition of pBR322 vector sequences to give cointegrate-like structures (Fig. 4, B-D) (Ref. 3,4,15). To explain this, Hirschel et al., (15) proposed that Tn5 transposes by a rolling circle replicative mechanism (Fig. 3), and that at the termination of replication the recA+ protein regulates how DNA strands are to be broken and then joined to give simple insertions or cointegrate products. In formulating their model they unfortunately did not realize that dimeric and higher oligomeric forms of pBR322::Tn5 plasmids arise by homologous recombination and generally comprise at least one-fourth of any plasmid population in recA⁺ cells. In reconstruction experiments using dimeric pBR322::Tn5 plasmids in recA⁻ cells as transposition donors we found that about half of the transposition products contained vector as well as Tn5 sequences (4). However, the

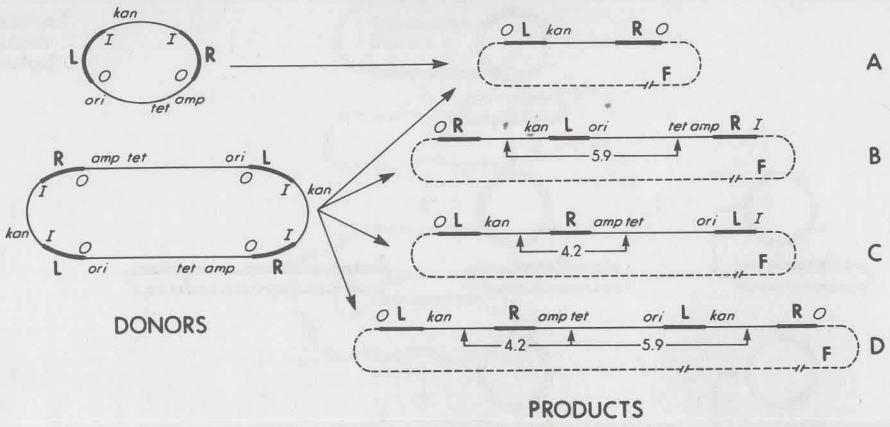


Fig. 4. Test of conservative model for IS50 and Tn5 transposition using monomeric and dimeric pBR322::Tn5 plasmids in recombination-deficient bacteria. Thickened lines designated L and R correspond to IS50L and IS50R, respectively (adapted from Ref. 4).

complete dimeric plasmid was never transposed (see Fig. 4). Thus, the cointegrate-like transposition products obtained in recA$^+$ cells probably result from: (i) simple insertion of segments of dimeric and larger plasmids, and (ii) from homologous recombination following transposition. There is no convincing evidence that Tn5 ever transposes by a replicative process (4).

The Tn3 element which is unrelated to Tn5, and which transposes replicatively and generates cointegrates, encodes a protein called resolvase which catalyzes site-specific recombination between direct repeats of Tn3. Because of this protein cointegrates generated by Tn3 transposition are unstable in recA$^-$ as well as in recA$^+$ cells, unless the gene for resolvase or its site of action is mutant (2,10).

In contrast to the instability of plasmids containing direct repeats of Tn3, dimeric pBR322::Tn5 plasmids and more complex structures containing direct repeats of IS50 or Tn5 (Fig. 4, 5) were easily generated in recA$^+$ cells and stably maintained in recA$^-$ cells (4,13,14,19). We concluded, therefore, that Tn5 does not encode a resolvase (4,13,14). But, a recent report had concluded that the transposase of Tn5 does in fact possess a resolvase activity (22). This new interpretation was based on the studies of a particular plasmid with direct repeats of IS50 elements, and on the rapid accumulation of bacterial subclones harboring recombinant derivatives of this plasmid. The accumulation of recombinants was said to be blocked by mutations in the transposase gene, and thus it was argued that the transposase protein encoded by Tn5 could also function as a resolvase (22).

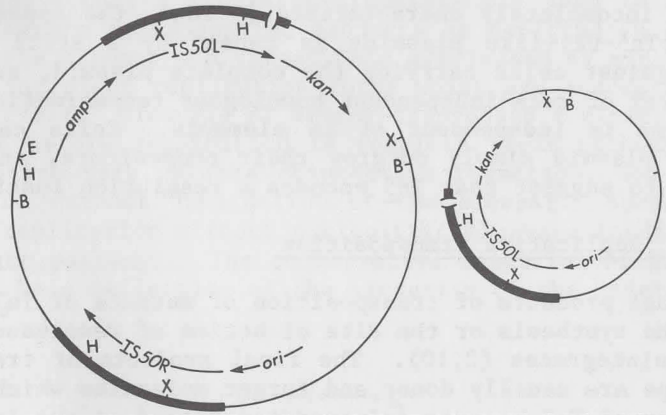


Fig. 5. The structure of pTn5-DR1 (left) and a plasmid (right) derived from it by recombination between directly repeated IS50 elements (thickened lines) in vivo. The letters B, E, H, and X indicate the sites cleaved by the restriction endonucleases BamHI, EcoRI, HindIII, and XhoI, respectively (adapted from Ref. 13). Only IS50R encodes transposase in the strains used in our studies. IS50L contains an ochre mutant allele of the transposase gene as a result of a single base-pair substitution 112 bp from the inside end of the 1534-bp element indicated by ( ) (adapted from Ref. 18).

Because this interpretation contradicts the body of evidence suggesting that Tn5 does not encode a resolution function, we re-evaluated the role of transposase in the breakdown of plasmids containing directly repeated copies of IS50. Our analysis indicates that the interpretation of Zupancic et al. (22) is incorrect; there is no evidence that Tn5 encodes a resolution function. We used pTn5-DR1 (Fig. 5; Ref. 13), a plasmid containing direct repeats of IS50 generated from pBR322::Tn5-wild-type by the in vitro rearrangement of BamHI fragments. Occasional breakdown of pTn5-DR1 in the absence of recA function might be expected because homologous recombination does occur in RecA$^-$ cells, although at a very low rate (8). We observed that cells carrying pTn5-DR1 form extremely small colonies [and Zupancic et al. (22) noted a similar slowing in growth rate caused by their plasmid]. We have found, in addition, that: (i) growth rate differences between cells carrying pTn5-DR1 and its recombinant are strongest on kanamycin-containing agar, (ii) recombinants are only rarely obtained after growth on kanamycin-free agar, (iii) poor growth with kanamycin is not corrected by insertions of Tn5-wild-type elsewhere in the genome, and (iv) simple point mutations in the transposase gene of pTn5-DR1 (at the XhoI and HindIII sites marked X and H, respectively, in IS50R in Fig. 5) do not relieve the growth defect (17). [The "transposase" mutations, which Zupancic et al. (22) believed stabilized their plasmid, were

complex and incompletely characterized.] Thus, the apparent instability of pTn5-DR1-like plasmids is caused by a still mysterious selection against cells carrying the complete plasmid, superimposed on a low level of recA-independent homologous recombination which in other systems is independent of IS elements. Cells carrying the recombinant plasmid simply outgrow their progenitors, and there is no evidence to suggest that Tn5 encodes a resolution function (17).

Tn3 and Mu: Replicative Transposition

The final products of transposition of mutants of Tn3 defective in either the synthesis or the site of action of resolvase are nearly always cointegrates (2,10). The final products of transposition Tn3-wild-type are usually donor and target molecules which each contain one copy of Tn3 because cointegrates formed as the initial products of transposition are broken down by resolvase. Similarly the induction of lytic growth of Mu prophages leads to transposition concomitant with Mu replication, and cointegrates equivalent to those mediated by Tn3 are formed whenever Mu transposes between replicons (7). These findings led Arthur and Sherratt (2) and Shapiro (20) to propose the bidirectional replicative transposition model (Fig. 2).

IS1 and Mu: Rolling-Circle-Replicative Transposition

In contrast to the cointegrates found after induction of Mu prophages, only single copies of Mu are found inserted after phage Mu infection (11). In addition, in the case of IS1 which is unrelated to Mu, only a fraction of transpositions result in cointegrates (9). Since neither Mu nor IS1 seem to encode an active resolution function, the findings of simple insertions appear to be inconsistent with the bidirectional replicative transposition model. This inconsistency led Harshey and Bukhari (11) and Galas and Chandler (9) to propose the rolling circle model (Fig. 3). The rolling circle model assumes that all transposition is replicative, and provides an explanation for simple (noncointegrate) transposition products as well as cointegrates as primary products of transposition.

There has always been reason to question the assumption that all transposition is replicative which underlies the rolling circle model. Mu insertion after infection has recently been shown to be conservative not replicative (1,16). In addition, we have examined the products of transposition from monomeric and dimeric forms of pBR322::Tn9 (IS1) plasmids (6), and our results indicate that the noncointegrate products of IS1 transposition probably do not arise by a rolling circle mechanism. Rather, with IS1, as with IS50, the simple insertions appear to arise by events in which the element failed to duplicate. Thus, recent results with Mu and with IS1 call for the consideration of new models, ones in which transposition is sometimes conservative and sometimes replicative.

We propose that a single intermediate can lead to cointegrate or noncointegrate transposition products as depicted in Fig. 6. In this model the element and target DNAs are joined by single strands, generating incipient replication forks which are susceptible to nucleolytic attack. Cleavage of vector-transposable element junctions at any time before replication is complete (left pathway) destroys the replication fork, blocks cointegrate formation, and results in transposition without duplication of the element. Successful completion of replication without nucleolytic cleavage leads to cointegrates (right pathway). The conservative insertion of Mu after infection may be a reflection of the linearity of the injected Mu DNA,

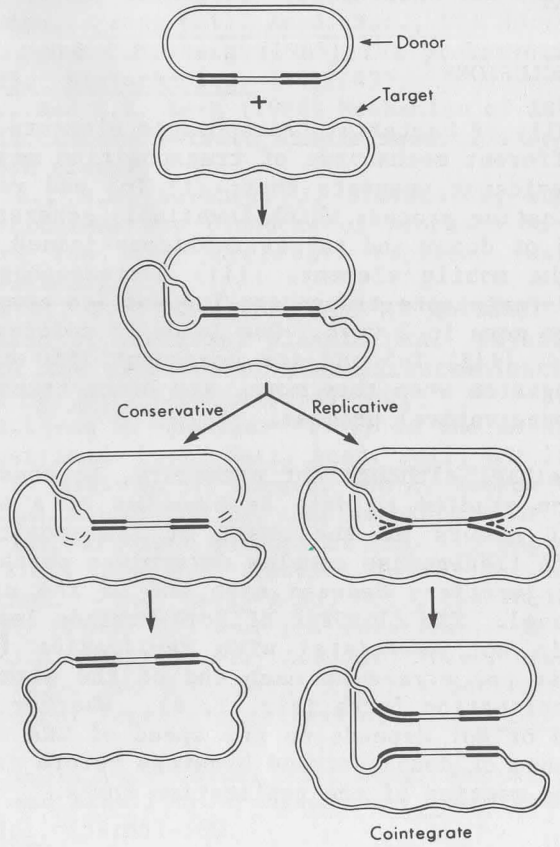


Fig. 6. A model of transposition in which replication (right fork) and nucleolytic cleavage of the donor-element junction (left fork) compete. Shown on the left is the case in which cleavage prior to the onset of replication results in a simple (noncointegrate) structure. However, breakage any time before replication is completed would have the same formal consequences (adapted from Ref. 6).

or of a different balance of the Mu insertion proteins. It is not necessary to invoke a rolling circle model to explain simple insertions as well as cointegrates.

It is possible that even IS50 and Tn5 transpose via the mechanism outlined in Fig. 6, with the caveat that the structure of the transposase leads invariably to destruction of the vector-element junctions prior to completion of replication, and hence to apparently conservative transposition. However, since IS50 appears to be unrelated to the other elements discussed here, it need not transpose by exactly the same mechanism. We still favor our original idea, that double-strand breaks at the vector-element junctions at the start of transposition irrevocably commits IS50-related elements to insertion without replication.

SUMMARY AND CONCLUSIONS

The diversity of bacterial transposable elements indicates that a number of different mechanisms of transposition may have evolved. The available evidence suggests that: (i) Tn3 and related elements move by a replicative process which invariably generates cointegrate DNAs consisting of donor and target replicons joined by direct duplications of the mobile element. (ii) Bacteriophage Mu, and the chloramphenicol-resistance transposon Tn9 and its component IS1 elements, appear to move in 2 ways. One leads to cointegrates, and the other does not. (iii) Tn5 and its component IS50 elements do not generate cointegrates when they move, and hence transpose by a non-replicative (conservative) process.

It is possible, although not necessary, to consider all types of transposition studied to date as branches of a single pathway. One branchpoint occurs at the onset of transposition, when the structure of the transposase complex determines whether one or both strands at the junctions between each end of the element and its vector are cleaved. The cleavage of both strands leads to transposition which is not associated with replication (Fig. 1). The cleavage of just one strand at each end of the element causes the formation of replication forks (Fig. 2, 6). Whether the element is then duplicated or not depends on the speed of DNA synthesis relative to the chance of double-strand breakage before transposition is completed by the meeting of the replication forks.

ACKNOWLEDGEMENTS

I am grateful to C.M. Berg for critical readings of this manuscript. This work was supported by U.S. Public Health Research Grants 5 Rol AI 14267 and 1 Rol AI 18980.

REFERENCES

1. Akroyd, J.E., and N. Symonds (1983) Evidence for a conservative pattern of transposition of bacteriophage Mu. Nature 303:84-86.
2. Arthur, A., and D.J. Sherratt (1979) Dissection of the transposition process: A transposon-encoded site-specific recombination system. Mol. Gen. Genet. 175:267-274.
3. Berg, D.E. (1977) Insertion and excision of the transposable kanamycin resistance determinant Tn5. In DNA Insertion Elements, Plasmids and Episomes, A.I. Bukhari, J.A. Shapiro, and S.L. Adhya, eds. Cold Spring Harbor Press, New York, pp. 205-212.
4. Berg, D.E. (1983) Structural requirement for IS50-mediated gene transposition. Proc. Natl. Acad. Sci., USA 80:792-796.
5. Berg, D.E., and C.M. Berg (1983) The prokaryotic transposable element Tn5. Biotechnology 1:417-435.
6. Biel, S.W., and D.E. Berg (1984) Mechanism of IS1 transposition in E. coli: Choice between simple insertion and cointegration Genetics (in press).
7. Chaconas, G., R.M. Harshey, J. Sravetnick, and A.I. Bukhari (1981) Predominant end products of prophage Mu DNA transposition during the lytic cycle are replicon fusions. J. Mol. Biol. 150:341-359.
8. Doherty, M.J., P.T. Morrison, and R. Kolodner (1983) Genetic recombination of bacterial plasmid DNA: Physical and genetic analysis of the products of plasmid recombination in Escherichia coli. J. Mol. Biol. 167:539-560.
9. Galas, D.J., and M. Chandler (1981) On the molecular mechanism of transposition. Proc. Natl. Acad. Sci., USA 78:4858-4862.
10. Gill, R., F. Heffron, G. Dougan, and S. Falkow (1978) Analysis of sequences transposed by complementation of two classes of transposition deficient mutants of Tn3. J. Bact. 136:742-756.
11. Harshey, R.M., and A.I. Bukhari (1981) A mechanism of DNA transposition. Proc. Natl. Acad. Sci., USA 78:1090-1094.
12. Heffron, F. (1983) Tn3 and its relatives. In Mobile Genetic Elements, J.A. Shapiro, ed. Academic Press, New York.
13. Hirschel, B.J., and D.E. Berg (1982) A derivative of Tn5 with direct terminal repeats can transpose. J. Mol. Biol. 155:105-120.
14. Hirschel, B.J., D.J. Galas, D.E. Berg, and M. Chandler (1982) Structure and stability of transposon 5-mediated cointegrates. J. Mol. Biol. 159:557-580.
15. Hirschel, B.J., D.J. Galas, and M. Chandler (1982) Cointegrate formation by Tn5, but not transposition, is dependent on recA. Proc. Natl. Acad. Sci., USA 79:4530-4534.
16. Liebhart, J., P. Ghelardini, and P. Paolozzi (1983) Conservative integration of bacteriophage Mu DNA into pBR322 plasmid. Proc. Natl. Acad. Sci., USA 79:4361-4366.

17. Phadnis, S.H., and D.E. Berg (1984) RecA-independent recombination between direct repeats of IS50 elements is not mediated by the IS50-encoded transposase (submitted for publication).
18. Rothstein, S.J., and W.S. Reznikoff (1981) The functional differences in the inverted repeats of Tn5 are caused by a single base pair nonhomology. Cell 23:191-199.
19. Sasakawa, C., J.B. Lowe, L. McDivitt, and D.E. Berg (1982) Control of transposon Tn5 transposition in Escherichia coli. Proc. Natl. Acad. Sci., USA 79:7450-7454.
20. Shapiro, J.A. (1979) Molecular model for the transposition and replication of bacteriophage Mu and other elements. Proc. Natl. Acad. Sci., USA 76:1933-1937.
21. Shapiro, J.A., ed. (1983) Mobile Genetic Elements. Academic Press, New York.
22. Zupancic, T.J., S.L. Marvo, J.H. Chung, E.G. Peralta, and S.R. Jaskunas (1983) RecA-independent recombination between the direct repeats of IS50. Cell 3:619-637.

INSERTION OF TRANSCRIPTIONAL ELEMENTS OUTSIDE THE REPLICATION REGIO

CAN INTERFERE WITH REPLICATION, MAINTENANCE, AND STABILITY OF

ColE1-DERIVED PLASMIDS

Hermann Bujard, Dietrich Stueber, Reiner Gentz,
Ulrich Deuschle, and Ursula Peschke

Molekulare Genetik
Universität Heidelberg
INF 230, 69 Heidelberg
Federal Republic of Germany

INTRODUCTION

The primary goal of our work is to contribute to a better un derstanding of structure-function relationships of nucleotide se quences defining promoters in the E. coli system. Since strong unregulated promoters can be assumed to exhibit pertinent structura features most clearly, we investigated, in vitro, more than 50 pro moters for their ability (a) to compete for the binding of RNA poly merase (an indication of their "signal strength") and (b) to produc RNA ("overall efficiency" of a promoter) under conditions of limit ing amounts of RNA polymerase. Promoters fulfilling these require ments best were found primarily in the genome of coliphage T5 (1) Some of these promoters show extremely high rates of complex forma tion with RNA polymerase (1,2) and at the same time were identifie as being very efficient initiators of RNA synthesis in vitro and i vivo (1,3).

For a better understanding of such transcriptional signals their sequences had to be cloned to (a) obtain amounts sufficien for biochemical and physical studies in vitro and (b) investigat their properties in vivo under genetically comparable conditions However, none of the commonly available cloning vehicles permitte

---

Present Address: F. Hoffmann-La Roche and Co. AG, 4002 Basel Switzerland.

the stable integration of these promoter classes (3), and it became obvious that intense transcriptional activities can interfere in various ways with plasmid maintenance and structural stability. We therefore constructed 2 families of plasmid vectors which not only allow us to stably integrate promoters independently of their strength, but also enabled us to identify some of the problems which heavily transcribed regions can cause to ColE1-type vector systems (3,4).

The principle of these plasmid systems is shown in Fig. 1 and their main properties are described in the corresponding legend.

Copy Number and Plasmid Maintenance

The pLBU3 vector (Fig. 1) permitted the cloning of various phage T5 promoters (3). However, those found to be most efficient in vitro appeared to be either not clonable or they caused a drastic reduction in the copy number, often accompanied with the loss of the entire plasmid upon prolonged propagation of the host cells. Since the reason for this phenomenon could have been an overproduction of tet gene products as well as a possible transcriptional readthrough into the replication region, we developed the pDS1 plasmid family (4). As has been shown (4), integration of a Xba I linker into the Pvu II site of pBR322 [which destroys the coding sequence of the ROP-protein (5)] increased the plasmid copy number 2- to 3-fold, indicating that a possible overproduction of this regulator protein by readthrough from upstream promoters can reduce plasmid copy number and subsequent maintenance. This is demonstrated best by the fact that T5 promoter P_{207} is stably maintained in pDS1, albeit at a reduced copy number (Fig. 2). Insertion of an efficient terminator at site 1 or 2 of pDS1 (Fig. 1), however, increases the copy number again (Fig. 2), showing that transcriptional readthrough into the replication region by itself has a profound effect on plasmid replication. We have not analyzed whether this is due to an overproduction of RNA I (6), a possible "mechanical" interference with the transcriptional unit for RNA II (7), or both.

Strong transcriptional readthrough into the replication region towards the primer promoter (producing RNA II, Ref. 7) also interferes with plasmid replication. Whereas promoters up to 4 times the efficiency of the β-lactamase promoter (P_{bla} or P_3, in Ref. 8) can be tolerated, sequences which exceed this promoter's efficiency 20-fold or more cannot stably be integrated in place of P_{bla} under conditions where translation of β-lactamase m-RNA would have been impossible (9,10).

In summary, for the construction of highly efficient transcriptional units within plasmid vectors carrying ColE1-type replicons, it has to be taken into account that promoter efficiency has to be "balanced" for the unit to be stably maintained. This can be achieved best by inserting compatible transcriptional signals, preferably in addition to repressible promoter elements.

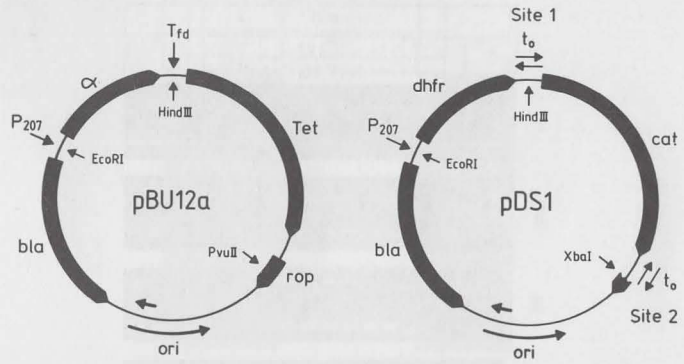


Fig. 1. Genetic and physical map of pBU12a and pDS1. Both plasmids contain 2 easily assayable functions which can be brought under the control of a single promoter (P) but which can also be separated by the insertion of a terminator (T). These functions are for the pBU series: β-galactosidase activity (via α-complementation α) and tetracycline resistance (tet); for the pDS series: trimethoprime (via dihydrofolate reductase, dhfr) and chloramphenicol (chloramphenicol acetyl transferase, cat) resistance. Both plasmids confer ampicillin resistance (via β-lactamase, bla) and carry the ColE1 replication region of pBR322 (ori). A major difference of the 2 plasmid systems is the presence of the intact rop gene in pBU12a and a second site for the integration of a terminator (site 2) in pDS1. The direction of transcription of the coding sequences is indicated. Positions and directions of transcription for RNA II (primer RNA) and RNA I are represented by arrows. The physical maps of both plasmids are not drawn to scale. Integration of coliphage fd terminator into pBU12a results in pLBU3 which was used for the cloning of coliphage T5 promoters (3). Insertion of terminators like t_o of coliphage λ (4) at site 1, 2 or at both sites makes this plasmid an even better cloning vehicle for promoter cloning. Promoters and other signals can be placed in front of the dhfr gene into various restriction sites (only the EcoRI site is shown here). Whereas the cat gene carries its own translational initiation site, there is no translation of dhfr-specified message unless a ribosomal binding site has previously been fused to the dhfr gene.

The Influence of Terminator Position on Plasmid Dimerization in pDS1

As shown above, placing the terminator t_o in position 1 or 2 of pDS1 carrying the phage T5 promoter P_{207} re-establishes the copy number to that of the promoter-free plasmid (Fig. 2). However, in analyzing the ratio of monomeric to dimeric forms of the plasmid, it can be clearly recognized that t_o in position 1 increases the number

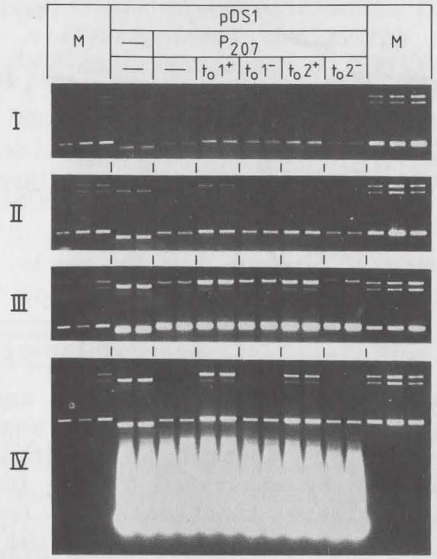


Fig. 2. Electrophoretic analysis of pDS1 derivatives at various cell stages (for details, see Ref. 4). The roman numerals designate the nucleic acid preparations from cells of stage I to IV, M are concentration standards. The duplicate probes can be identified by the headings whereby the following abbreviations are used: P_{207}, coliphage T5 promoter P_{207} inserted into the EcoRI site of pDS1 (Fig. 1); t_o1^+ and t_o1^- describes the insertion of the coliphage λ terminator t_o at site 1 in functional (1^+) and nonfunctional (1^-) orientation, respectively; the same holds for t_o2^+ and t_o2^-. The first band on the top of each gel represents the dimeric, the lowest the monomeric form of the plasmid. In comparing the dimeric forms in stage II and stage IV, the influence of the positioning of t_o can be seen most clearly.

of dimeric structures. This is confirmed by the experiment shown in Fig. 3. Since it has been shown that multimerization decreases the maintenance of ColE1-derived plasmids (11), these observations show that even such seemingly trivial topographic rearrangements of otherwise identical signals can affect the stable propagation of a vector. This result is puzzling in a special way: proposing that heavily transcribed regions may have some impact on a plasmid's replication, a size reduction of this region by moving t_o from position 2 to 1 would be expected to have the opposite effect.

Increase of Recombinational Events in Heavily Transcribed Regions

We have constructed a pDS1 derivative containing 3 identical T5 promoter sequences tandemly fused around 100 bp apart (12). Since a

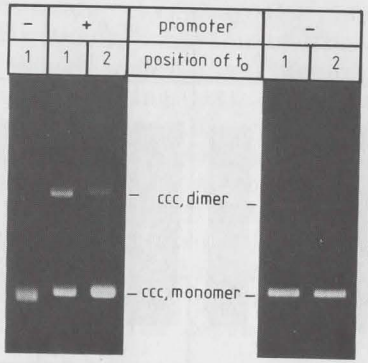


Fig. 3. Influence of the positioning of terminators on the dimerization of pDS1. The different probes of the electrophoretic analysis in agarose gels can be identified by the heading of the figure. The promoter used is P_{207} from coliphage T5. The increase in plasmid dimerization in presence of P_{207} and terminator t_o in position 1 (Fig. 1) is seen in lane 2 (from the left).

battery of 3 strong promoters was assumed to possibly create problems, the promoters were previously fused to a lac operator sequence. In addition, terminator t_o was placed in position 1 and no ribosomal binding site was present to allow the production of DHFR. This construct is perfectly stable if grown in the presence of sufficient amounts of lac repressor (Fig. 4). Upon induction with IPTG, colonies appear at high rates wherein the plasmid has lost one or 2 promoters by homologous recombination (evidenced by fine mapping of restriction sites and sequence analysis). Transferring the plasmid into rec A or rec F strains markedly increased the stability of the construct (Fig. 4). There are 2 interpretations to those results:

- Increased intensity of transcription has an impact on the frequency of recombinational events whereby it is not clear whether the tandemly repeated promoter regions represent a special situation, or whether similar results can be obtained with tandemly repeated sequences further downstream of the transcriptional initiation sites.

- There is a normal low frequency of elimination of promoters but, under condition of transcription, plasmids with less intense transcription (i.e., lower promoter number) have a replicative advantage.

In favor of the first interpretation, we have never observed differences in copy numbers between plasmids in which promoters differ severalfold in their efficiency--as long as translation of the overproduced m-RNA and readthrough into the replication region is prevented.

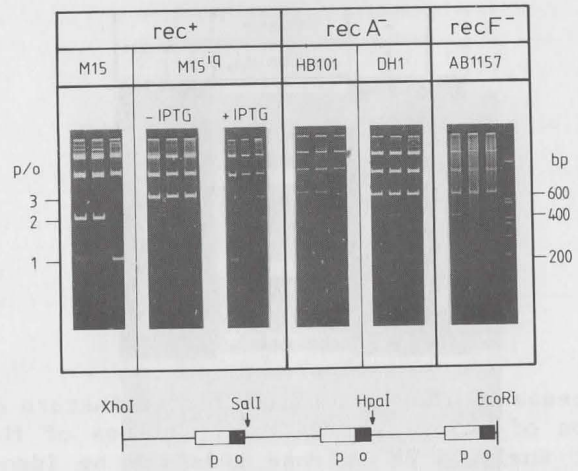


Fig. 4. Stability of a tandemly repeated promoter/operator construct. The upper part of the figure shows the electrophoretic analysis (6% polyacrylamide) of pDS1 containing t_o in position 1 (Fig. 1) and the triple promoter/operator construct shown in the lower part of the figure. The distance between the promoter/operator regions is 100 bp. The host system and the conditions of growth (± IPTG) of the different plasmid isolates can be taken from the headings. At the left side the numbers of promoter/operator regions within the different isolates are indicated, which are 600, 400, or 200 bp in length, respectively, as pointed out at the left side of the figure. The cultures were grown to $OD_{600} \triangleq 2.0$ in LB medium.

Plasmid Carried Translational Initiation Signals and the Possible Limits of the Translational Capacity of the Host System

The pDS1 system carrying a proper promoter insertion permits quantification of different translational initiation signals by inserting the unknown signal in front of the dhfr gene. The resulting polycistronic m-RNA (terminator in position 2) codes for 2 proteins: DHFR and CAT. Since the amount of CAT depends on its original translation initiation signal, the ratio of CAT to DHFR can be used to compare translation initiation signals of unknown activity (13). The products of both indicator genes (dhfr and cat) are tolerated by the cell in concentrations of up to 50% of the total soluble protein if only one of the genes is translated or up to 30% each if both gene products are allowed to be synthesized. Using this system, we have identified some unusually efficient translational initiation signals. Some of these, however, can only be cloned in presence of a strong promoter if an efficient transcriptional terminator is placed in position 2 of the plasmid (Fig. 1). Attempts to integrate

such signals into plasmids carrying the identical terminator in position 1 failed. Our interpretation of these experiments is that the amount of DHFR produced under these conditions may become toxic to the cell. However, having stoichiometric amounts of CAT-ribosomal binding sites (terminator in position 2) competing with the signal in front of the dhfr gene appears to allow the survival of the cell. Since under these conditions around 90% of the newly synthesized proteins consist of CAT and DHFR, these experiments seem to indicate that, by use of the proper expression signals in multi-copy plasmids, at least the translational machinery of the host can be exhausted at certain stages of growth. Consequently, the stable propagation of a plasmid can also depend on physiological parameters of this type.

CONCLUDING REMARKS

The results described here were obtained as "side products" in a program in which plasmids are merely being used as tools to produce and to study signals of gene expression in vitro and in vivo. Even in such simple constructs as discussed here, where we know the DNA sequences and the topography of the main functions, it appears that we often cannot predict the outcome of seemingly homologous experiments, and puzzling questions accumulate. They indicate that even such straightforward artificial plasmid constructs cannot be looked at in a static way. Instead, they appear as dynamic entities in which, for example, the extent of transcription in various regions may represent only one such variable to be taken into account should such a system become predictable.

REFERENCES

1. Gabain, A.V., and H. Bujard (1979) Interaction of E. coli RNA polymerase with promoters: The relative rates of complex formation and decay with promoters of several phage and plasmid DNA's. Proc. Natl. Acad. Sci., USA 76:189-193.
2. Brunner, M. (1984) Untersuchungen zur Wechselwirkung von E. coli RNA-Polymerase mit Promotoren. Diplomarbeit, Universität Heidelberg, 69 Heidelberg, FRG.
3. Gentz, R., A. Langner, A.C.Y. Chang, S.N. Cohen, and H. Bujard (1981) Cloning and analysis of strong promoters is made possible by the downstream placement of an RNA termination signal. Proc. Natl. Acad. Sci., USA 78:4936-4941.
4. Stueber, D., and H. Bujard (1982) Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. EMBO 1:1399-1404.
5. Cesarini, G., M.A. Muesing, and B. Polisky (1982) Control of ColE1 DNA replication: The Rop gene product negatively affects transcription from the replication primer promoter. Proc. Natl. Acad. Sci., USA 79:6313-6317.

6. Muesing, M., J. Tamm, H.M. Shepard, and B. Polisky (1981) A single base-pair alternation is responsible for the DNA overproduction phenotype of a plasmid copy-number mutant. Cell 24:235-242.
7. Tomizawa, J., and T. Itoh (1981) Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. Proc. Natl. Acad. Sci., USA 78:6096-6100.
8. Stueber, D., and H. Bujard (1981) Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci., USA 78:167-171.
9. Deuschle, U. (1984) Entwicklung einer Methode zur quantitativen Bestimmung der in vitro- als auch in vivo-Effizienzen von Transkriptionssignalen aus dem E. coli-system. Diplomarbeit, Universität Heidelberg, 69 Heidelberg, FRG.
10. Deuschle, U., R. Gentz, and H. Bujard (unpublished results).
11. Summers, D.K., and D.J. Sherrat (1984) Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability. Cell 36:1097-1103.
12. Peschke, U. (1984) Konstruction von tandemartig angeordneten Promoter/Operator sequenzen zur analyse der wechselwirkung diser sequenzen mit RNA-polymerase bzw. Repressoren. Diplomarbeit, Universität Heidelberg, 69 Heidelberg, FRG.
13. Bujard, H., C. Baldari, M. Brunner, U. Deuschle, R. Gentz, J. Hughes, W. Kammerer, and D. Stueber (1983) Integration of efficient promoters of the E. coli system into plasmid vectors. In Gene Amplification and Analysis, T.S. Papas, M. Rosenberg, and J.C. Cirikjian, eds. Elsevier/North Holland, New York, Vol. 3, pp. 65-88.

STUDIES ON THE TRANSPOSITION OF IS1

Michael Chandler[1] and David J. Galas[2]

[1]Centre de Recherche de Biochimie et Génétique Cellulaire du C.N.R.S.
118, Route de Narbonne
31062 Toulouse Cedex, France

INTRODUCTION

The insertion sequence, IS1, is among the smallest insertion elements known. It is a member of a class of elements which includes IS10, IS50, and IS903 which are frequently found in pairs flanking a nontransposable DNA segment. In concert, the 2 flanking elements are able to promote transposition of the entire structure. This type of transposable element has been called a compound or composite transposon. The best characterized IS1-flanked compound transposon is perhaps Tn9 in which 2 directly repeated copies of IS1 flank a 1.1-kb segment of DNA which encodes chloramphenicol acetyltransferase (CAT) (1). Unlike most other elements of this class, however, IS1 is present in the chromosomes of a variety of enterobacteria, in some cases in many copies (2).

IS1 also forms part of several clinically important multiple antibiotic-resistance plasmids where it flanks, in direct repeat, the majority of the resistance genes and delimits a structure having a molecular length of the order of 20 kb (the resistance determinant or r-det) (3-5). The r-det is itself composed of a series of intercalated transposable elements. The flanking IS1 elements give the resistance genes a structural autonomy enabling the r-det to behave as an integral unit. Indeed, derivatives of the resistance plasmid R100.1 have been isolated in Escherichia coli from which supernumerary circular copies of the r-det are generated (9). This process

---

[2] Molecular Biology University of Southern California, Los Angeles, California 90089-1481.

requires the host recA and recBC gene products and can be repressed in trans by the presence of a closely related plasmid in the same cell. The r-det can also undergo tandem duplication (amplification) (6) and transmission to other replicons, by a mechanism which depends largely on homologous recombination between IS1 elements, but may also involve IS1-mediated transposition at a very low frequency (7,8). The insertion sequence IS1 therefore provides a large degree of fluidity for the antibiotic-resistance genes carried by these plasmids, a feature which has undoubtedly contributed to their rapid dissemination. The presence of multiple copies of this insertion sequence in the host chromosome might also be expected to provide similar flexibility within the host genome.

In spite of, or perhaps because of, its presence in the chromosome of most laboratory strains of E. coli, the analysis of the behavior and structure of IS1, and of the organization and expression of IS1-encoded genes is not as advanced as is that of some other elements of this class such as IS50 and IS10. Transposition of IS1 has many aspects in common with that of other insertion elements: it generates a duplication in the target DNA at the point of insertion (10-12) and it induces replicon fusions (cointegrate formation) (13), deletion, and inversion of DNA segments (14,15). For certain transposons, for example those of the Tn3 family, cointegrate formation is an integral part of the major transposition pathway. It is thought to be a replicative mechanism in which the element is copied into its target site while a copy is retained at the original site (see Ref. 16 for review). Where donor and recipient replicons are circular plasmids, the resulting cointegrate structures are composed of fused copies of the donor and recipient separated at each junction by a copy of the element, in a directly repeated configuration. Subsequent recombination between the 2 elements (resolution) completes the transposition, resulting in the regeneration of the donor replicon and of the recipient which then carries a single copy of the element. On this scheme deletions and inversions are viewed as the result of intramolecular transposition.

While IS1 mediates cointegrate formation, several lines of evidence suggest that this is not the sole, nor the most efficient, pathway for transposition of this element (17,18). Thus, in contrast to Tn3-mediated cointegrates, Tn9 (IS1)-mediated cointegrates are relatively stable and, under some circumstances, their frequency of formation is lower than that of Tn9 transposition (17). Cointegrates mediated by other members of this class of transposon are also relatively stable. This suggests that cointegrates are not intermediates in the transposition of these elements, and has led to the proposal that they are alternative end products to direct transposition of the element in the transposition process (18). Although several models have been proposed to explain the relationship between cointegrate formation and direct transposition (for reviews, see Ref. 16 and 19), insufficient experimental evidence is available at present to allow definite conclusions to be drawn.

IS1-flanked compound transposons exhibit at least one other interesting property. Comparison of the transposition frequency of a series of IS1-flanked transposons from a lambda prophage to 2 different target plasmids revealed an approximately exponential decrease in transposition frequency with increasing size (20). Similar results have been obtained with other transposons, in particular Tn10 (21). The simplest interpretation of this length dependence is that some aspect of the transposition process occurs in a processive manner. This could occur during replication of the element as we initially suggested (18) or at any stage in the process which involves one-dimensional tracking of the transposition complex across the entire compound transposon.

In the following, we present a summary of the results of an ongoing study of some of the factors which affect the transposition activity of IS1 and the relative frequencies of cointegrate formation and transposition. We also present an analysis of the structure and coding capacity of the element. The implications of these and other results on the evolution and maintenance of IS1-flanked compound transposons are discussed.

THE EXPERIMENTAL SYSTEM

The experimental system employed throughout these studies uses 2 plasmids: a conjugative plasmid, pOX38, derived from the plasmid F which has been deleted for all known insertion sequences carried by the parent plasmid (22); and the nontransmissible multicopy plasmid, pBR322 or its derivatives. The plasmid pOX38 itself carries no selectable marker and in certain experiments we have therefore used a derivative of this plasmid, pOX38Km, which carries a HindIII fragment of Tn5 specifying resistance to kanamycin cloned into the unique HindIII site of pOX38. The transposon under study can be placed on either of the 2 plasmids by transposition or by in vitro techniques. Using this system, transposition from pBR322 derivative plasmids can be simply monitored by pOX38-mediated transfer of the transposon-associated resistance gene. Cointegrate formation can likewise be monitored by pOX38-mediated conjugal transfer of pBR322 to a suitable recipient. Such transfer can only occur after prior fusion of the 2 plasmids mediated by a transposable element carried by one or the other (17,23). In all experiments, the donor strain harboring a pOX38 and pBR322 derivative plasmid carried a recA mutation. In experiments in which the structures of various transposition products were to be analyzed, the independence of each event was ensured by the use of a single exconjugant colony from each of a series of independent matings using separate isolated colonies of the donor strain.

MODULATION OF COINTEGRATE-FORMING ACTIVITY BY TRANSCRIPTION

In view of the fact that the flanking insertion elements associated with compound transposons retain some of their functional independence, it is interesting to consider the factors which determine the relative frequencies of transposition events involving either the entire compound transposon or the flanking sequences. Since IS1 has no selectable phenotypic marker, it is difficult to measure its frequency of transposition directly. We have therefore made use of the fact that Tn9 induces cointegrates in which the element involved in the fusion (either of the individual IS1 elements or the entire Tn9) is duplicated and appears as a directly repeated copy at the junctions between the 2 replicons (Fig. 1). The relative frequency of appearance of cointegrates in which Tn9 is repeated at both junctions or in which Tn9 occurs at one and IS1 at the other can be used as a measure of the relative activities of the entire Tn9 and its component IS1 elements in cointegrate formation. This in turn presumably reflects the relative transposition frequency of the components.

In the following, the IS1 elements flanking Tn9 have been called IS1A and IS1B, respectively (17). The relative position of the elements with respect to transcription of the CAT gene is shown

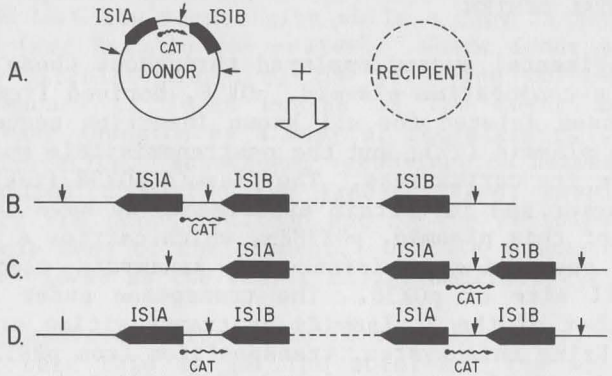


Fig. 1. Cointegrate formation by Tn9. This figure shows schematically the way in which various cointegrate structures are formed: ▬▶, IS1; ∿∿, CAT gene transcript; ↓, restriction site used for structure determinations. These structures were analyzed by digestion with a restriction enzyme which cleaves within the CAT gene of Tn9 (EcoRI) and within the donor plasmid. The use of a particular IS1-element is characterized by which of the specific IS1-carrying fragments of the donor replicon (A) is no longer present in the cointegrate. A fuller explanation of this analysis will be found in Ref. 17 and 23. (B) IS1B-mediated; (C) IS1A-mediated; (D) Tn9-mediated.

in Fig. 1. Cointegrates generated by Tn9 or by one or the other flanking IS1 element can be simply distinguished by digestion with suitable restriction enzymes (17,23). The analysis of 40 cointegrates generated between pOX38::Tn9 and pBR322 indicated that the overall frequency of utilization of the IS1's was about 10 times that of Tn9 (17). Surprisingly, however, one of the flanking IS1 elements, IS1A (Figs. 1,2), was almost 10-fold more active than the other. Thus the activities of the flanking IS1 elements are either inherently different, as is the case with the flanking elements of Tn5 and Tn10, or the difference in activity is due to differences in the nucleotide sequence environment of the 2 elements.

In order to distinguish between these 2 possibilities, we cloned Tn9 (isolated as a BglII-XhoI fragment together with about 150 bp of surrounding DNA of the pOX38 donor plasmid) from pOX38::Tn9 into various positions and orientations within the plasmid pBR322. We subsequently analyzed the structure of cointegrates generated between these pBR322-Tn9 derivative plasmids and pOX38 (23). A summary of these results is shown in Fig. 2. It can be seen that the relative frequency with which IS1 elements are used in the formation of cointegrates varies enormously from plasmid to plasmid. There is, however, a strong correlation between the relative activity of the IS1 elements and the orientation of Tn9 with respect to the known transcription units of pBR322. The plasmids pXB, pR2.4, and pR2.5 carry Tn9 cloned between the BamHI and SalI sites (pXB), or into the EcoRI site in either orientation (pR2.4 and

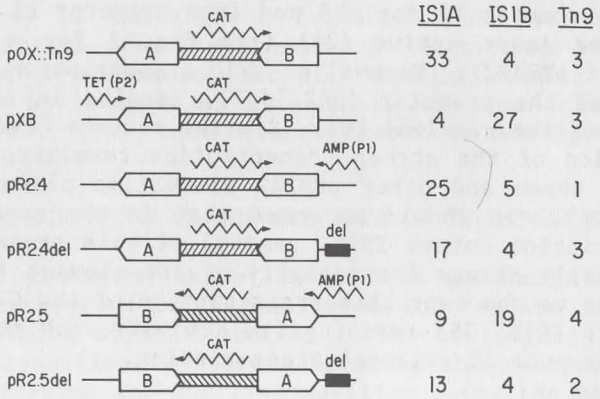


Fig. 2. Relative frequency of IS1A, IS1B and Tn9-mediated cointegrates. This figure shows the relative frequency of 3 types of cointegrate generated by pOX38::Tn9 and by various pBR322:Tn9 derivative plasmids. ∿∿, transcripts; ■, deletion of promoter P1; pXB; Tn9 cloned between the BamHI and SalI sites of pBR322 (see Fig. 3); pR2.4 and pR2.5, Tn9 cloned into the EcoRI site of pBR322. The construction of these plasmids is described in Ref. 23.

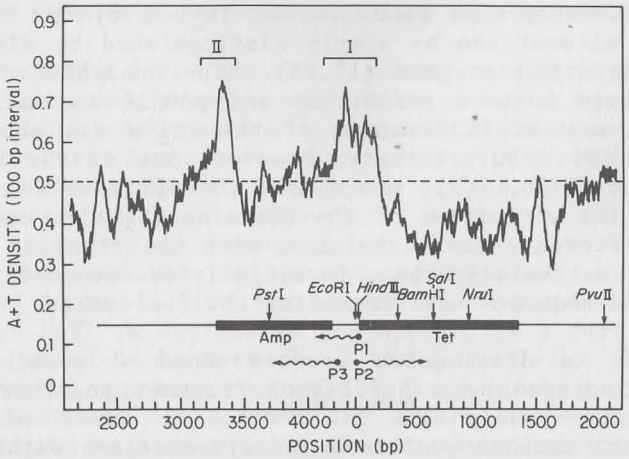


Fig. 3. Physical and genetic map of plasmid pBR322. This figure shows: the position of the promoters P1, P2, and P3; the direction of transcription from these promoters, ⟿; location of the Ampicillin and Tetracycline resistance genes, ▬; and the position of the sites of cleavage of the various restriction enzymes used in the generation of the derivative plasmid described here. Also included is the relative proportion of A+T base pairs in the molecule using a window of 100 bp.

pR2.5). In all 3 plasmids, the IS1 proximal to an oncoming transcript (from promoter P2 for pXB and from promoter P1 for pR2.4 and pR.2.5) is the least active (24) (see Fig. 3 for a physical and genetic map of pBR322). Removal of this transcription, for example, by deletion of the promoter (pR2.5del1), results in an increase in the activity of the proximal IS1. Similar results have been obtained by insertion of the strong transcription terminator of phage T4 gene 32 into these and other pBR322 derivative plasmids (Ref. 23; data not shown). It should be noted that in the case of pR2.4 in which transcription enters IS1B, removal of this transcription does not significantly change the activity of the element (23). This is presumably due to the fact that transcription of the CAT gene of Tn9 continues into IS1B (25) reducing its activity with respect to IS1A even in the absence of external transcription.

Thus the difference in activity of the IS1 elements is determined largely by external transcription and is not inherent to the element. The effect of transcriptional inhibition can also be seen in the frequency of cointegrate formation. The frequencies of IS1-mediated cointegrate formation between pOX38Km and in vitro deletion derivatives of pR2.4 and pR2.5 retaining a single IS1 are shown in Fig. 4. Both pR2.4IS1 and pR2.5IS1 show an approximately 10-fold

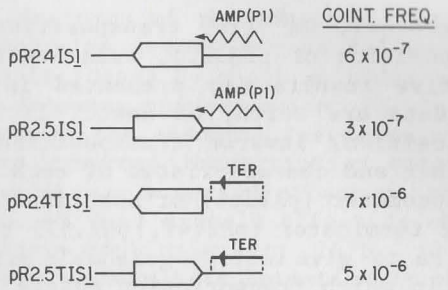


Fig. 4. Cointegrate formation frequency of pBR322::IS1 derivative plasmids. Plasmids carrying a single copy of IS1 were derived from the respective Tn9-carrying plasmids by in vitro deletion using the unique BstEII site in the IS1 elements (23).

The strong transcription terminator of phage T4 gene 32 was introduced into these plasmids on a HindIII/BamHI fragment of pBH857 (57) by substitution between the pBR322 HindIII and BamHI sites (Fig. 3) (23).

Cointegrate formation frequencies were measured using pOX38Km as a target molecule and the results are the mean values of 10 independent experiments (23). Transcription from promoter P1 (Fig. 3) is indicated, ⟿; and the terminator from T4 gene 32 is indicated, ←.

lower frequency of cointegrate formation than do derivatives in which transcription from the promoter P1 is blocked by substitution of the T4 gene 32 terminator for P1. Similar results have recently been obtained by Machida et al. (25). In the case of the Tn5, Sasakawa et al. (26) have demonstrated an analogous effect on transposition.

EFFECTS OF TRANSCRIPTION ON THE SPECTRUM OF TRANSPOSITION PRODUCTS

The data presented above demonstrate an inhibitory effect of external transcription on IS1-mediated cointegrate formation. In order to determine whether similar inhibitory effects are also exerted on transposition, we have determined the relative frequencies of cointegrate formation and transposition using the various pBR322-Tn9 derivative plasmids as donors and pOX38Km as a target molecule. Transposition from the circular donor plasmids can in principle occur in 2 senses: transposition of Tn9 itself or transposition of the pBR322 segment of the plasmid (inverse transposition) (27-29). Experimentally, all 3 types of event can be simply monitored by determining the relative frequency of transfer of various combinations of pBR322-Tn9-associated markers: Cm^r together with Ap^r

and/or Tc^r (cointegrates), Cm^r (Tn9 transposition), and Ap^r and/or Tc^r (inverse transposition of pBR322), relative to that of pOX38Km alone. Representative results are presented in Fig. 5. Several features of these data are worthy of note: 1) the relative frequencies of transposition, inverse transposition, and cointegrate formation are distinct and characteristic of each of the 3 plasmids; 2) deletion of the promoter (pXBdel) or substitution of the promoter with the T4 gene 32 terminator (pR2.4T, pR2.5T) results in convergence of these spectra to give indistinguishable patterns; 3) The plasmids pXB and pR2.5 in which transcription enters IS1A exhibit quite different patterns. These results demonstrate that transcription influences all 3 types of transposition events.

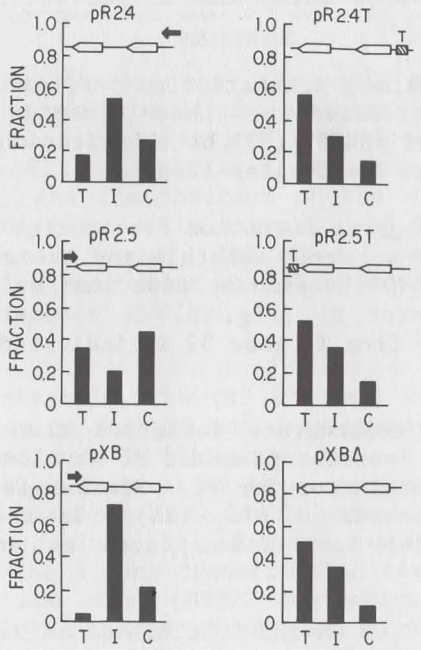


Fig. 5. Spectra of Tn9-mediated recombination products. Tn9-mediated transposition (T), inverse transposition (I) and cointegrate formation (C) were measured as described in the text, using pOX38Km as a target molecule. The relative frequencies are represented here as fractions of the total observed recombination products. The donor plasmids are indicated above each histogram and the environment of the Tn9 represented: ➡, transcription; ▨, transcription terminator from T4 gene 32 (57); the vertical line in the pXBΔ panel indicates the deletion endpoint near the left end of Tn9. The construction of these plasmids is described in Ref. 23.

The principal features of the absolute frequencies (Tab. 1) of each of the transposition events are as follows: 1) the overall frequency of events increases by about an order of magnitude following suppression of incoming transcription; 2) In all 3 cases, there is a marked increase in the absolute frequency of transposition; 3) In both cases where external transcription enters IS1A (pR2.5 and pXB), its removal also has a marked effect on the frequency of inverse transposition; 4) Only a small effect is observed in the case of pR2.4 in which transcription enters IS1B. 5) In all 3 plasmids only a small effect on cointegrate formation is observed.

A coherent explanation for the above results is that transcription into either end of IS1 reduces the probability that it is recognized (or bound) by a transposition complex. The magnitude of this effect must be comparable at either end. Thus the cointegrate formation frequencies of pR2.4IS1 and pR2.5IS1 are similar and increase to a similar extent when transcription inhibition is relieved. The effect seems to be transmitted through the element to some extent since both transposition (using the "outer" ends of the IS1

Tab. 1. Frequencies of transposition, cointegrate formation, and inverse transposition.

	TRANSP.	COINT.	INV.	TOTAL
pR2.4	2.3×10^{-7}	3.5×10^{-7}	6.9×10^{-7}	1.3×10^{-6}
pR2.4T	4.2×10^{-6}	1.0×10^{-6}	2.2×10^{-6}	7.5×10^{-6}
pR2.5	3.2×10^{-7}	4.0×10^{-7}	1.9×10^{-7}	9.0×10^{-7}
pR2.5T	3.8×10^{-6}	1.1×10^{-6}	2.6×10^{-6}	7.5×10^{-6}
pXB	3.9×10^{-8}	1.7×10^{-7}	5.7×10^{-7}	7.8×10^{-7}
pXBdel	4.2×10^{-6}	9.7×10^{-7}	2.9×10^{-6}	8.1×10^{-6}
pBRIS1.11 (monomer)	--	2.0×10^{-7}	--	--
pBRIS1.11 (dimer)	--	3.1×10^{-7}	--	--

The plasmids and methods used in measuring these frequencies have been described (Ref. 23 and text). The plasmid pBRIS1.11 carries an IS1 insertion at pBR322 coordinate 3270 and was isolated from a cointegrate between pOX38::Tn9 and pBR322 as described. Following the introduction of the pBR322 derivative plasmids by transformation in the construction of the donor strains, the presence of monomeric and dimeric plasmids was ascertained by agarose gel electrophoresis of small preparations of plasmid DNA from the transformants. The numbers are the means of ten independent measurements.

elements flanking Tn9) and inverse transposition (using the "inside" ends) are affected by the same transcript (see pXB and pR2.5). In this light it is perhaps significant that no sequences characteristic of strong transcription terminators are obvious in the sequence of IS1 (30,31), neither are all IS1 insertions polar (32,33). This raises the possibility that some transcription could continue through the element to affect the distal end.

The effect of release of transcription inhibition in the case of pR2.4 is interesting. The relatively large increase in transposition accompanied by a small increase in inverse transposition suggests that the "outside" end of IS1B is affected to a greater extent by incoming transcription than is the "inside" end. Presumably, transcription of the CAT gene into the inside end of IS1B is the major factor in determining the activity of this end (25).

The consistently smaller effect of transcription on cointegrate formation in all 3 plasmids is not unexpected. Transposition is presumably determined by the activity of both flanking IS1 elements whereas cointegrate formation can occur using one or the other. Transcription entering one IS1 element would thus inhibit transposition of the entire compound transposon while it would reduce cointegrate formation only by the contribution of the inhibited element.

Finally, an interesting possibility suggested by these results is that translation of the incoming transcript also inhibits transposition activity. An increase of over 2 orders of magnitude in transposition frequency is observed following relief of transcription inhibition by deletion of the Tet gene promoter in pXB (pXBdel) whereas only a 10-fold increase is observed for pR2.5 (pR2.5T). Since pXB carries Tn9 cloned within the Tet gene of pBR322, the oncoming Tet transcript is presumably also translated. For pR2.5 in which Tn9 is cloned into the EcoRI site, no translation of the incoming transcript would be expected. The difference in behavior of pXB (pXBdel) and pR2.5 (pR2.5T) could reflect a supplementary inhibition in pXB due to translation. In contrast to the transcription effect, the hypothetical translation effect would seem not to be transmitted through the element since the change in inverse transposition in pXB is similar to that found with pR2.5. Translation inhibition of events requiring the "inside" ends would thus be lower than that of events requiring the "outside" ends. Here it should be noted that nonsense codons are found at both ends of IS1 in all 3 reading frames, preventing translation into the element. We are investigating the effect of removing translation of the truncated Tet gene in the behaviour of pXB. It indicates that the complex changes in the spectrum of products can be explained by simple changes in the activities of the ends.

The above descriptive treatment is of necessity oversimplied and is presented only to provide a framework. A detailed quantitative interpretation of these results has been performed but presentation of this analysis would be too lengthy to include here (Galas and Chandler, in preparation).

REPLICATIVE AND NONREPLICATIVE EVENTS

One of the strongest pieces of evidence that at least some transpositional events are replicative is the appearance of cointegrates, since the cointegrate of donor and target replicon carries 2 copies of the transposon. There is good evidence of replication in the case of Tn3 (34). Analysis of adjacent deletions mediated by IS903 provides direct evidence that these events also occur by a replicative mechanism (35). On the other hand, the observation of the strong dependence of cointegrate formation on recA function in the case of Tn5 (36) may indicate that simple transposition from dimers of the donor replicon are involved in cointegrate formation, rather than replicative fusion from a monomeric donor molecule. Cointegrate formation from dimeric pBR322::Tn5 plasmid molecules has been reported to be significantly greater than from monomeric molecules (37). While there is some question of the above interpretation of the Tn5 observations, the results do suggest that Tn5 and IS1 may function differently (Chandler and Galas, in preparation). We have measured the frequency of IS1- mediated cointegrate formation from monomeric and dimeric donor molecules and found them to be indistinguishable (Tab. 1). This indicates that cointegrate formation is primarily replicative for IS1 since the element must be replicated in the case of the monomeric donor.

INSERTION SPECIFICITY

The influence of the donor molecule on the outcome of transposition described above prompted us to investigate more closely features of the target molecule which might also have an influence.

It has been noted that IS1 exhibits a strong preference for regions of DNA which are rich in A+T (38,39); that insertion in the lacZ gene of E. coli occurs close to a region of homology with the extremity of the element; and that there is a strong preference for cleavage of the target DNA next to a G C base pair (38). It is probable, therefore, that several factors act in concert to determine the final choice of target site, perhaps on different scales of sequence length.

In order to investigate in a more systematic manner the various factors that influence the choice of target site, we have analyzed the distribution of IS1 insertions in the fully sequenced plasmid pBR322 (40,41) and certain derivatives. Insertions of IS1 were isolated from cointegrate plasmids generated by fusion between pBR322

and pOX38::Tn9 following mating to a suitable recombination- proficient recipient strain. Selection was for the transfer of the pBR322-associated tetracycline resistance marker in most cases, and thus precluded the isolation of insertions within this gene. Since such cointegrates are relatively stable, we have adopted the following strategy for isolating pBR322:IS1 derivative plasmids from them. Cointegrates are isolated in a recombination-proficient (recA$^+$) host and plasmid DNA prepared from such strains (42) following copy number amplification. This treatment results in an enrichment for pBR322 derivative plasmids in the population, i.e., for those derivatives which have excised from the cointegrate in general by homologous recombination between the directly repeated copies of the flanking transposable element. The recombination can give rise to both pBR322::Tn9 and pBR322::IS1 derivatives (see Fig. 1). The plasmid DNA was then used to transform E. coli C600 to tetracycline resistance. Crystal violet was included in the plates to screen for chloramphenicol-sensitive transformants presumably carrying a plasmid with a single IS1 (43). Plasmid DNA isolated from chloramphenicol-sensitive transformants by the rapid lysis method (42) was screened for size by agarose gel electrophoresis and plasmids exhibiting the size expected for pBR322:IS1 were retained for further analysis. Digestion with either HinfI and HinfI+PstI or PstI and PstI+EcoRI was performed to determine the orientation and position of IS1 in each of the pBR322::IS1 plasmids. Typical results are shown in Fig. 6 and a summary of these results is presented in Fig. 7.

The plasmid pBR322 carries 2 regions of high A+T content (Fig. 3). One, region I, is situated (approximately) between coordinates 4150 and 150 and includes the promoter P3 of the β-lactamase gene. The other, region II, is located between coordinates 3200 and 3400 and covers part of the 3' end of the β-lactamase gene. It can be seen from the results presented in Fig. 7a that just over half (15/25) of the IS1 insertions occur in region I while 6 of the insertions fall in region II and the remaining 4 fall in the relatively A+T-rich β-lactamase gene. Since the region(s) of pBR322 which exhibit strong homology with the ends of IS1 (7/7 base matches) are located at coordinates 406, 415, 1871, and 2084, some distance from the preferred insertion site, this result suggests that A+T richness is more important in determining the sites of insertion than are regions exhibiting homology with the end of the element. More interestingly, of the 15 insertions within region I, 13 occur in one orientation. The insertions shown in this figure were isolated following selection for the transfer of pBR322-associated tetracycline resistance. We have also analyzed 9 plasmid derivatives which were isolated in the same experiment, but following selection for transfer of pBR322-associated ampicillin (carbenicillin) resistance so that the tetracylcine resistance marker was no longer excluded as a target. All 9 isolates proved to carry IS1 inserted in region I and 8 of these were in the prevalent orientation.

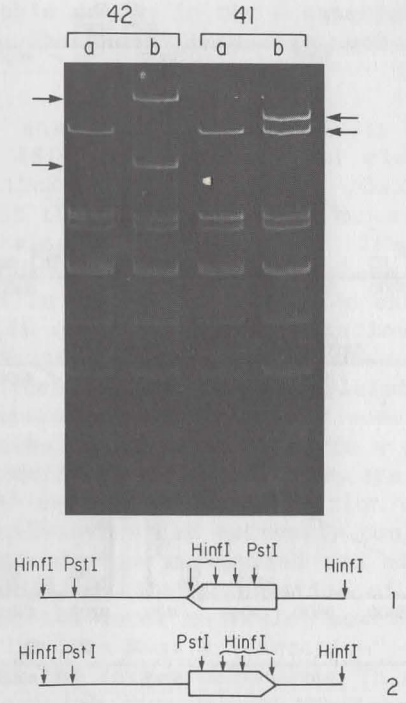


Fig. 6. Analysis of insertion specificity into pBR322 and pKH47. This figure illustrates the analysis of IS1 insertions into the plasmid pKH47, using HinfI + PstI (a) and HinfI alone (b). The plasmids, 41 and 42, carry IS1 insertions into the major "hot spot" region I (Fig. 3) in opposite orientations. For insertions in one orientation, both junction fragments (→) are cleaved by PstI (plasmid 1) while in the other, only one of these fragments is cleaved (plasmid 2).

The position of the relevant restriction sites for IS1s in the region I "hotspot" are shown schematically below.

Thus, of 24 insertions isolated in region I, 21 proved to occur in one orientation. It is also clear from this result that there is no "hotspot" in the Tc^r gene.

The preferential orientation of insertion of IS1 in region I of pBR322 derivative plasmids is further illustrated by the results presented in Fig. 7b. In this series of experiments, 31 independent pOX38::Tn9-mediated IS1 insertions were isolated in the plasmid pKH47. This plasmid is a derivative of pBR322 which carries a re-

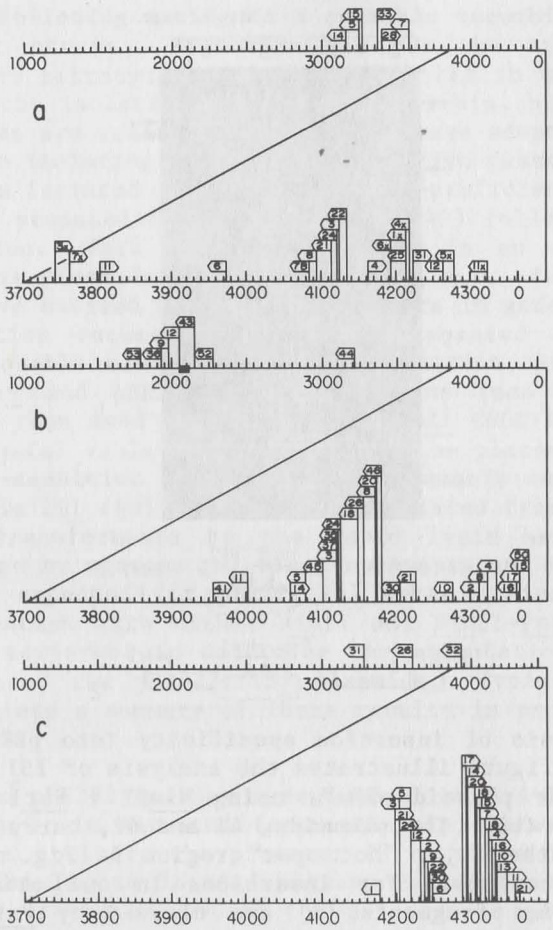


Fig. 7. Distribution of IS1 Insertions into pBR322 and pKH47. a) Insertions of IS1 (Tn9) in pBR322. b) Insertions of IS1 (Tn9) in pKH47. c) Insertions of IS1 (Tn2671del72) in pBR322. ■, 60 bp poly (dA) ·poly(dT) insert.

gion of poly dA:dT residues introduced by polynucleotide kinase tailing (44). The plasmid used in our experiments was found to carry an insertion of approximately 60 bp within the unique PvuII site of pBR322. Note that, of 31 insertions mapped, only 1 occurs in region II of the plasmid while 6 occur in the neighborhood of the poly A:T segment. Thus, as expected, the synthetic poly dA:dT region attracts insertions of IS1 (45). Region I of the plasmid remains, however, the most favored region. Twenty-two of the 31 insertions are found in this region. More significantly, perhaps, almost all (21 of the 22 insertions) are found in the preferred orientation observed in the pBR322 target. Thus, of a total of 46 insertions occurring in this region in the 2 experiments, 42 are observed in one orientation. The probability that such a distribution occurs by chance is low.

We have also analyzed the specificity of insertion using Tn2671.72, another IS1-flanked transposon closely related to Tn9 (46). Using pOX38::Tn2671.72 in place of pOX38::Tn9 in the mating system, we found that the distribution of insertions into pBR322 was the same as that obtained with pOX38::Tn9. The results of an analysis of 27 IS1 insertions into pBR322 are shown in Fig. 7c. Twenty-four insertions fall in region I. They also exhibit a strong orientation preference (21 occur in one orientation). This orientation is opposite, however, to that observed in the case of pOX38::Tn9 donor. It is clear, then, that the donor plasmid/transposon can determine the orientation of insertion. A possible explanation for the orientation reversal is that it reflects a difference in the activities of the extremities of the IS1 elements in the 2 transposons due to the effect of transcription inhibition described previously. For example, the "activity" of an extremity could represent the probability that the extremity is recognized and nicked prior to insertion into its target site. The orientation of insertions in pBR322 cannot depend only on the donor molecule, however. An observed orientation preference implies that a "direction" must be distinguished both in the donor and the target molecule. This could be determined by an oriented recognition sequence in the target, or a directional process such as transcription or replication. We are at present investigating these possibilities.

While it is reasonable to expect that the strong inhibitory effect of transcription could provide one of the required preferences in the donor, it is not at all clear what factors could provide the strand preference in the target. To test one possibility, that the transcription through region I from promoter P1 was responsible, we inserted the T4 gene 32 transcription terminator in the EcoRI site of pBR322 to block this transcription and isolated a set of IS1 inserts into this plasmid using both donor plasmids. This collection exhibited the same orientation preference as before (data not shown), indicating that transcription is not the orientation-determining factor in the target.

STRUCTURE AND ORGANIZATION OF IS1

Analysis of the nucleotide sequence of IS1 has shown that the extremities are composed of 2 imperfectly homologous segments of about 35 bp inverted with respect to each other (30,31). To begin to determine the role of these extremities, we have constructed a group of pBR322-derived plasmids. These carry an IS1 element or a 76 bp fragment of one end of the element cloned into the PvuII site of pBR322 and a second IS1 cloned into the EcoRI site in one or the

other orientation. These constructions should yield transposons carrying the pBR322 Tet gene. In order to improve selection for the transposon at low copy number, part of the intervening Tet gene (between the HindIII and NruI sites; Fig. 3) of these 4 plasmids was replaced by a 1.8 kb Hind III/PvuII fragment of the plasmid pKp6 (47) specifying resistance to streptomycin and spectinomycin (P. Gamas, unpub. data).

The frequency of transposition (Sp^r, Sm^r) onto, and of cointegrate formation (Sp^r, Sm^r, Ap^r) with pOX38Km as determined for each of these plasmids is presented in Fig. 8. It can been seen that the plasmids pOC17 and pOC18 which each carry 2 complete copies of IS1 exhibit similar transposition and cointegrate formation frequencies. This indicates that there is no dramatic difference in the activity of the 2 extremities of IS1. Since we do not at present know the transcription pattern of the inserted Sp^rSm^r fragment, we are unable to ascertain the effects of transcription on the activity of the IS1 elements. It is therefore difficult to interpret the differences between the cointegrate formation frequency and that of transposition observed for both plasmids.

While we do not yet fully understand the reason for the differences in activity of pOC15 and pOC16, it is clear from these results that 76 bp at one extremity of IS1 are sufficient for transposition of the synthetic compound transposon. It should be noted here that

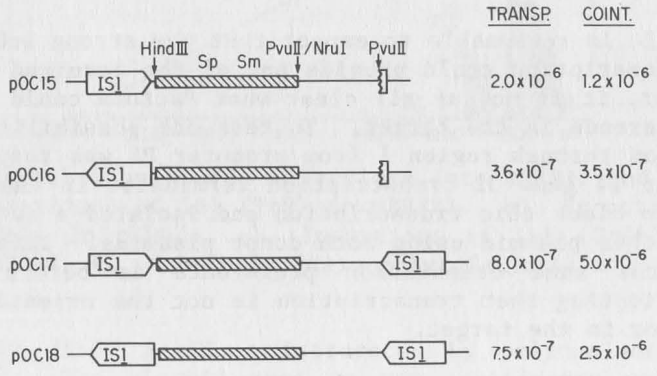


Fig. 8. Transpostion of Sm^r/Sp^r transposons. Transposition and cointegrate formation frequencies of the 4 transposons which form part of a pBR322 based replicon was measured using pOX38Km as a target plasmid. The construction of these plasmids is briefly described in the text and will be described in detail elsewhere. {, 76 bp extremity of IS1 delimited by the IS1 PvuII site; ▨, spectinomycin, streptomycin resistance fragment. The frequencies are the means of 10 independent experiments.

inverse transposition of Ap^r from these plasmids occurred at a frequency of $<10^{-9}$ (the lower limit of detection in these experiments) while it occurred at about 10^{-7} in the case of pOC17 and pOC18. This demonstrates that the extremities of IS1 used in a transposition event must be in the correct relative orientation for successful transposition. Experiments to define the minimum size of the extremity for successful transposition are in progress.

Sequence analysis has also shown that IS1 cannot code for any large polypeptides but indicates that it could code several small polypeptides (Fig. 9). As pointed out by Rak et al. (48) and others (49), many insertion elements have coding frames arranged in a characteristic manner: a large open reading frame coding in one direction and a significantly shorter overlapping open frame coding in the opposite direction with an identical frame configuration (codons in phase). IS1 is peculiar in this respect in not exhibiting the long reading frame. It is like the others, however, in having 2 opposite-strand, aligned reading frames (A and D) in the left half of the molecule (Fig. 9). Because of their conservation in 2 iso-

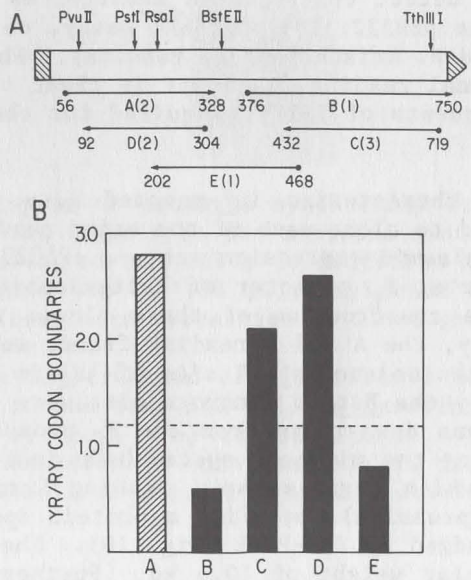


Fig. 9. Organization of IS1. A. Map of IS1 showing relevant restriction sites, and several major open reading frames (ORF) together with their reading phases. The inverted homologous extremities are also indicated. B. Histogram of the number of codon boundaries found with the pyrimidine-purine (YR) character to those with the purine-pyrimidine (RY) character. The expected ratio if codon usage were completely random is 1.2 (52).

insertion elements of IS1, it has been suggested that frames A and B are good candidates for encoding functional IS1 polypeptides (50). However, theoretical considerations of the codon boundary preference exhibited by each potential open reading frames ha led to the suggestion that, while frames A, C and D show a strong positive correlation with the preferred pyrimidine/purine (YR) codon boundary often noted in coding regions (51), B does not. The degree of preference for YR codon boundaries is shown for the open reading frames in IS1 (greater than 50 amino acid equivalents in length) in Fig. 9b. This had led to the suggestion that B may not code for a functional polypeptide (19,52).

Experimental definition of all the functional reading frames and the roles of their products is not yet available. By using S1 digestion at unique restriction cleavage sites in IS1, Ohtsubo and collaborators (53) have demonstrated that deletions in both the A (at PstI) and B (at TthIIII) reading frames render the element inactive. Results obtained in our laboratories in which we have used the Sm^r fragment of pKP6 to insert 14 bp of DNA [see Prentki and Krisch (47)] into several unique restrictions sites in IS1 (PvuII, 76 bp; RsaI, 209 bp; and BstEII, 328 bp; see Fig. 9a) are similar. We were unable to detect cointegrates mediated by these IS1 insertion mutants in the pBR322::IS1/pOXc38Km assay, to a lower limit of 10^{-9} (J. Robert and H. Krisch, unpub. results). Whatever the organization of functional reading frames it is clear that the integrity of much of the sequence of IS1 is required for the activity of the element.

In order to characterize IS1-encoded gene products we have therefore attempted to clone each of the major reading frames, A, B, C, and D, in the plasmid expression vector, pPLC236 (54), under the control of the strong P_L promoter of bacteriophage λ. A detailed description of the construction of these clones will be presented elsewhere. Briefly, the A and D reading frames were separated from B and C by using the unique BstEII site of IS1 in the cloning. The 2 halves defined by the BstEII cleavage site were cloned separately in both orientations downstream from the P_L promoter. Initial results obtained using the minicell system have indicated that one of the clones (that which carries the A reading frame in the correct orientation for expression) specifies a protein species of approximately 10 kd as judged by SDS-PAGE (Fig. 10). The protein InsA has an expected molecular weight of 10.6 kd. Further characterization of this and other potential IS1 gene products is in progress. Another open reading frame (designated F in Ref. 52) is correctly positioned to have produced a protein from the above construct. This hypothetical protein has not been observed.

We wish to point out that the InsA protein carries a region which exhibits a significant amino acid homology with the region of



Fig. 10. Autoradiogram of proteins synthesized from a clone carrying reading frame A in the minicell system. The isolation and labeling of minicells is described in Ref. 58. Electrophoresis was on 18% polyacrylamide SDS gels (58) and the proteins were visualized by fluorography.

The construction of the plasmid pOC 7 is described briefly in the text and will be described in detail elsewhere. a) pOC; 7b) vector pPLC236. The small abundant protein observed in both channels in presumably rop (59), the synthesis of which is probably driven by the cloned P_L promoter.

proteins such as cI and cro of bacteriophage λ, and CAP which are thought to be involved in the recognition of specific DNA sequences (55,56). InsA is also highly basic, a characteristic of many DNA binding proteins.

CONCLUSIONS

In the results presented above, we have provided evidence that transcription, and perhaps translation, into either end of IS1 inhibits its activity. For compound transposons such as Tn9, this effect not only determines which of the flanking IS1 elements is more likely to generate cointegrates, but may determine how insertion into a target region occurs, i.e., in one orientation or the other, and it determines the relative importance of fusions and transpositions proper as products of the transposition process. The fact that this effect is so marked suggests that it may play an important role in the evolution of IS1-flanked compound transposons and, together with some of the other properties of IS1 discussed above, it has interesting implications for the formation and maintenance of such transposons.

The preference of IS1 for A+T-rich regions would tend to increase the probability of insertion of the element in promoter or terminator regions, which are generally both A+T-rich (38,39). Such a tendency is illustrated by the preferential insertion of IS1 upstream from the β-lactamase and tet promoters of pBR322. This behavior increases the likelihood that the element will insert between transcription units. The inhibitory effect of transcription into IS1 means that elements which have inserted into a transcription unit will subsequently transpose at a reduced frequency and would tend to favor further transposition of elements which had inserted within nontranscribed regions. Inhibition by translation would further increase this selective effect. Finally, the decrease in the frequency of transposition with increasing length would be expected to favor relatively small compound transposons. Thus, in concert, these properties would tend to favor the formation and dissemination of compound transposons in which the flanking elements "isolate" relatively small regions of DNA carrying integral transcription units. Our studies have also demonstrated a rather dramatic preference in the orientation of IS1 insertions into the major hot spot of pBR322, a phenomenon which has not previously been noted (e.g., Ref. 38). While the sense of the orientation can be determined by the donor molecule, it is evident that the influence of the donor alone is not sufficient. The target region must also influence this asymmetry. One way in which this could occur is by donor modulation of the activities of the extremities of IS1 coupled with a preference for initial cleavage of one strand in the target region. The identical orientation preferences we obtained using a pBR322 derivative carrying terminator that stops transcription from P1 indicates that transcription through the hot spot is not a determining factor (data not shown). Alternative possibilities are that the direction of replication in the target, sequence-dependent DNA conformation, or oriented recognition sequence determine the orientation.

We are currently studying the structural and coding organization of IS1. Thus far, we have shown that the terminal 76 act as a functional end in transposition. From other experiments we have evidence for the synthesis of at least one IS1-coded protein in mini-cells. The biological activity of this protein remains unknown. The coding organization of IS1 is unusual among known insertion sequences in being composed of 5 major, overlapping reading frames (Fig. 9a). Among these major, open reading frames are 3 that exhibit a strong statistical preference for pyrimidine/purine (Y/R) codon boundaries, and 2 that do not (Fig. 9b). One of those showing this Y/R preference is insA, coding for the protein that is apparently expressed by one of our recombinant plasmids. Though hardly conclusive, a comparison of the amino acid sequence of InsA protein with that of several known DNA-binding proteins does suggest that there may be a structural motif in InsA similar to that of the sequence-specific binding regions of these known proteins. In particular, model building analysis has revealed that the carboxyterminal end of the protein may form a characteristic α-helix-turn-α-helix in which one face of the carboxyterminal helix is predominantly hydrophobic while the other is composed of predominantly polar residues. It remains for future studies to define the nature and functions of the IS1-coded proteins that are involved in the transposition activities of this element.

ACKNOWLEDGMENTS

We would like to thank A. Epstein, D. Rifat, B. Marty, and S. Wilkins for expert technical assistance, and P. Gamas, D. Zerbib, L. Kozar, and S. Bass for work on the various aspects of the work reported here. We would also like to thank P. Prentki, O. Fayet, G. Churchward, and R. Alazard for constructive discussions. The work reported here was supported by NIH Grant GM19036 to D.G., by grant 3.169.0.81 to L. Caro and M.C. from the Swiss National Science Foundation and by an "Action Thématique Programée" from the C.N.R.S. (France) to M.C.

REFERENCES

1. MacHattie, L.A., and J.B. Jakowski (1977) Physical Structure and deletion properties of the chloramphenicol resistance transposon Tn9 in phage lambda. In DNA Insertion Elements, Plasmids and Episomes, A.I. Bukhari, J.A. Shapiro, and S.L. Adhya, eds. Cold Spring Harbor Laboratory, New York, pp. 219-228.
2. Nyman, K., K. Nakamura, H. Ohtsubo, and E. Ohtsubo (1981) Distribution of the insertion sequence IS1 in gram-negative bacteria. Nature 289:609-612.
3. Ptashne, K., and S.N. Cohen (1975) Occurrence of insertion sequence (IS) regions on plasmid deoxyribonucleic acid as direct and inverted nucleotide duplications. J. Bact. 122:776.

4. Kopecko, D.J., J. Brevet, and S.N. Cohen (1976) Involvement of multiple translocating DNA segments and recombinational hotspots in the structural evolution of bacterial plasmids. J. Mol. Biol. 108:333-360.
5. Sharp, P.A., S.N. Cohen, and N. Davidson (1973) Electron microscope heteroduplex studies of sequence relations among plasmids of E. coli: Structure of drug resistance (R) factors and F factors. J. Mol. Biol. 75:235-255.
6. Rownd, R.H., and S. Mickel (1971) Dissociation and reassociation of RTF and r-determinants of the R factor NR1 in Proteus mirabilis. Nature 234:40-43.
7. Chandler, M., E. Boy de la Tour, D. Willems, and L. Caro (1979) Some properties of the chloramphenicol resistance transposon Tn9. Mol. Gen. Genet. 176:221-231.
8. Iida, S., C. Hanni, C. Echarti, and W. Arber (1981) Is the IS1-flanked r-determinant of the plasmid NR1 a transposon? J. Gen. Microb. 126:413-425.
9. Chandler, M., J. Sechaud, and L. Caro (1982) A mutant of the plasmid R100.1 capable of producing autonomous circular forms of its resistance determinant. Plasmid 7:251-262.
10. Grindley, N.D.F. (1978) IS1 Insertion generates a duplication of a nine base pair sequence at its target site. Cell 13:419-426.
11. Calos, M.P., L. Johnsrud and J.H. Miller (1978) DNA Sequence at the Integration Site of the Insertion Element IS1. Cell 13:411-418.
12. Iida, S., R. Marcoli, and T.A. Bickle (1981) Variant insertion element IS1 generates 8-base pair duplications of the target sequence. Nature 294:374-376.
13. Ohtsubo, E., M. Zenilman, and H. Ohtsubo (1980) Plasmids containing insertion sequences are potential transposons. Proc. Natl. Acad. Sci., USA 77:750-754.
14. Reif, H.J., and H. Saedler (1975) IS1 is involved in deletion formation in the gal region of E. coli K12. Mol. Gen. Genet. 137:17-28.
15. Cornelis, G., and H. Saedler (1980) Deletions and inversion induced by a resident IS1 of the lactose transposon Tn951. Mol. Gen. Genet. 178:376-374.
16. Kleckner, N. (1981) Transposable elements in procaryotes. Ann. Rev. Genet. 15:341-404.
17. Galas, D.J., and M. Chandler (1982) Structure and stability of Tn9-mediated cointegrates: Evidence for two pathways of transposition. J. Mol. Biol. 154:245-272.
18. Galas, D.J., and M. Chandler (1981) On the molecular mechanism of transposition. Proc. Natl. Acad. Sci., USA 78:4858.
19. Iida, S., J. Meyer, and W. Arber (1983) Procaryotic IS elements. In Movable Genetic Elements, J. Shapiro, ed. Academic Press, New York, pp. 159-221.
20. Chandler, M., M. Clerget, and D.J. Galas (1982) The transposition frequency of IS1-flanked transposons is a function of their size. J. Mol. Biol. 154:229-243.

21. Morisato, D., J.C. Way, H.-J. Kim, and N. Kleckner (1983) Tn10 transposase acts preferentially on nearby transposon ends in vivo. Cell 32:799-807.
22. Guyer, M.S., R.R. Reed, J.A. Steitz, and K.B. Low (1980) Identification of a Sex Factor Affinity Site in E. coli as γδ. Cold Spring Harbor Symp. Quant. Biol. 45:135-140.
23. Chandler, M., and D.J. Galas (1983) Cointegrate formation mediated by Tn9 II: Activity of IS1 is modulated by external DNA sequences. J. Mol. Biol. 170:61-91.
24. Stüber, D., and H. Bujard (1981) Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci., USA 78:167-171.
25. Machida, C., Y. Machida, H.-C. Wang, K. Ishizaki, and E. Ohtsubo (1983) Repression of cointegration ability of insertion element IS1 by transcriptional readthrough from flanking regions. Cell 34:135-142.
26. Sasakawa, C., J.B. Loew, L. McDivitt, and D.E. Berg (1982) Control of transposon Tn5 transposition in Escherichia coli. Proc. Natl. Acad. Sci., USA 79:7450-7454.
27. Chandler, M., E. Roulet, L. Silver, and L. Caro (1979) Tn10-mediated integration of the plasmid R100.1 in the bacterial chromosome: Inverse transposition. Mol. Gen. Genet. 173:23-30.
28. Reif, H.J. (1980) Genetic evidence for absence of transposition functions from the internal part of Tn981 a relative of Tn9. Mol. Gen. Genet. 177:667-674.
29. Rosner, J.L., and M.S. Guyer (1980) Transposition of IS1- bio-IS1 from a bacteriophage derivative carrying the IS1-cat-IS1 transposon (Tn9). Mol. Gen. Genet. 178:111-120.
30. Ohtsubo, E., and H. Ohtsubo (1978) Nucleotide sequence of an insertion sequence, IS1. Proc. Natl. Acad. Sci., USA 75:615-619.
31. Johnsrud, L. (1978) DNA sequence of IS1, a transposable element. Mol. Gen. Genet. 169:213-218.
32. Jaskunas, S.R., and M. Nomura (1977) Mapping of the ribosomal protein genes in E. coli by means of insertion mutations. In DNA Insertion Elements, Plasmids and Episomes, A.I. Bukhari, J.A. Shapiro, and S.L. Adhya, eds., Cold Spring Harbor Laboratory, New York, pp. 487-495.
33. Brewster, J.M., and E.M. Morgan (1981) Tn9 and IS1 inserts in a ribosomal ribonucleic acid operon of E. coli are incompletely polar. J. Bact. 148:897-903.
34. Grindley, N.F.D. (1983) Transposition of Tn3 and related transposons. Cell 32:3-5.
35. Wienert, T.A., N.A. Schaus, and N.D.F. Grindley (1983) Insertion sequence duplication in transpositional recombination. Science 222:755-765.
36. Hirschel, B.J., D.J. Galas, and M. Chandler (182) Cointegrate formation by Tn5, but not transposition, is dependent on recA. Proc. Natl. Acad. Sci., USA 79:4530-4532.

37. Berg, D.E. (1983) Structural requirements for IS30-mediated gene transposition. Proc. Natl. Acad. Sci., USA 80:792-796.
38. Galas, D.J., M.P. Calos, and J.H. Miller (1980) Sequence Analysis of Tn9 insertions in the lacZ gene. J. Mol. Biol. 144:19-41.
39. Meyer, J., S. Iida, and W. Arber (1980) Does the insertion element IS1 transpose preferentially into A+T rich DNA segments? Mol. Gen. Genet. 178:471-473.
40. Sutcliffe, J.G. (1978) Complete nucleotide sequence of the Escherichia coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43:77-90.
41. Peden, K.W.C. (1983) Revised sequence of the tetracycline resistance gene of pBR322. Gene 22:275-280.
42. Kline, R.D., E. Selsing, and R.D. Wells (1980) A rapid microscale technique for isolation of recombinant plasmid DNA suitable for framing. Plasmid 3:88-91.
43. Proctor, G.N., and R.H. Rownd (1982) Rosanilins: indicator dyes for cloramphenicol resistant enterobacteria containing chloramphenicol acetyltransferase. J. Bacter. 150:1375-1382.
44. Hyashi, K. (1980) A cloning vehicle suitable for strand separation. Gene 11:109-155.
45. Devos, R., R. Contreras, J. van Emmelo, and W. Fiers (1979) Identification of the translocatable element IS1 in a molecular chimera constructed with plasmid pBR322 DNA in which a bacteriophage MS2 DNA copy was inserted by the poly(dA)·Poly(dT) linker method. J. Mol. Biol. 128:621-632.
46. Arber, W., S. Iida, H. Jutte, P. Caspers, J. Meyer, and C. Hanni (1979) Rearrangements of genetic material in escherichia coli as observed in the bacteriophage P1 plasmid. Cold Spring Harbor Symp. Quant. Biol. 43:1197-1208
47. Prentki, P., and H.M. Kirsch (1982) A modified pBR322 vector with improved properties for the cloning, recovery, and sequencing of blunt-ended DNA fragments. Gene 17:189-196.
48. Rak, B., M. Lusky, and M. Hable (1982) Expression of two proteins from overlapping and oppositely oriented genes on transposable DNA insertion element IS5. Nature 297:124-128.
49. Klaer, R., S. Kuhn, H.-J. Fritz, E. Tillmann, I. Saint-Girions, P. Haberman, D. Pfeifer, and P. Starlinger (1980) The sequence of IS4. Cold Spring Harbor Symp. Quant. Biol. 45:215-224.
50. Ohtsubo, H., K. Nyman, W. Doroskiewicz, and E. Ohtsbuo (1981) Multiple copies of isoinsertion sequence IS1 in gram-negative bacteria. Nature 292:640-643.
51. Shepherd, J.C.W. (1981) Method to determine the reading frame of a protein from the purine pyrimidine genome sequence and its possible evolutionary justification. Proc. Natl. Acad. Sci., USA 78:1596-1600.
52. Galas, D.J., and T.F. Smith (1984) The relationship between codon boundaries and multiple reading-frame preferences: Coding organization of bacterial insertion sequences. Mol. Biol. Evol. 1:260-268.

53. Machida, Y., C. Machida, H. Ohtsubo, and E. Ohtsubo (1982) Factors determining frequency of plasmid cointegration mediated by insertion sequence IS1. Proc. Natl. Acad. Sci., USA 79:277-281.
54. Remaut, E., P. Stanssens, and W. Fiers (1981) Plasmid vectors for high efficiency expression controlled by the P1 promoter of coliphage lambda. Gene 15:81-93.
55. Anderson, W.F., Y. Takeda, D.H. Olendorf, and B.W. Matthews (1982) Proposed α-helical super-secondary structure associated with protein DNA recognition. J. Mol. Biol. 159:745-751.
56. Shaw, D.J., D.W. Rice, and J.R. Guest (1983) Homology between CAP and Fnr, a regulator of anaerobic respiration in Escherichia coli. J. Mol. Biol. 116:241-47.
57. Prentki, P., and H.M. Krisch (1984) In vitro insertional mutagenesis with a selectable DNA fragment. Gene (in press).
58. Roulet, E., B. Allet, and M. Chandler (1984) Cloning and expression of the Mu a gene. Gene (28:65-72).
59. Cesareni, G., M.A. Muesing, and B. Polisky (1982) Control of ColE1 DNA replication: The rop gene product negatively affects transcription from the replication primer promoter. Proc. Natl. Acad. Sci., USA 79:6313-6317.

ON THE TRANSPOSITION AND EVOLUTION OF Tn1721 AND ITS RELATIVES

Rüdiger Schmitt,[1] Sabine Mötsch,[1] Peter Rogowsky,[1]
Fernando de la Cruz,[2] and John Grinsted[3]

[1]Lehrstuhl für Genetik
Universität Regensburg
D-8400 Regensburg, Germany

INTRODUCTION

Transposition of Tn3-like elements involves cointegrate formation catalyzed by transposase and resolution of cointegrates catalyzed by resolvase (1).

The tetracycline-resistance transposon (tet) Tn1721 belongs to a group of Tn3-like elements also comprising Tn21 and Tn501 that encode interchangeable transposition functions but specify different resistances (2-4). We report here:

(i) on the arrangement of Tn1721-borne transposition genes (structure);

(ii) on transposase-mediated replicon fusion involving "single-ended" elements (mechanism); and

(iii) on the pedigree of Tn1721 and its relatives (evolution).

STRUCTURE OF Tn1721

Tn1721 consists of a minor transposon (Tn1722) encoding transposition functions and an amplifiable tet region encoding resistance

[2]Departamento de Bioquímica, Universidad de Santander, Santander, Spain; [3]Department of Microbiology, University of Bristol, Bristol BS8 1TD, United Kingdom.

(Ref. 2,5,6; Fig. 1). There are 3 38-base pair (bp) inverted repeats (IR) that allow transposition of either the whole element or the minor transposon alone. Transposition of Tn1721 generates 5-bp direct repeats at the target site (7). Amplification of the tet genes is strictly recA-dependent. Up to 9 tandem duplications of tet have been isolated and a positive gene dosage effect on tetracycline resistance has been shown (9).

Transposition genes are arranged in the order:

res (resolution site) - tnpR (resolvase) - tnpA (transposase)

with the latter genes having identical transcriptional polarity (from left to right; Ref. 10). Resolvase catalyzes the resolution of cointegrates by site-specific recombination at res. By binding to res that overlaps the tnpR promoter it represses its own transcription, but not that of the tnpA gene suggesting that they have independent promoters. When using transcriptional fusion to galactokinase, the tnpR promoter showed 40% of lac promoter efficiency, whereas the weak tnpA promoter was only detectable in the transposase-mediated transposition reaction. Transposase itself is temperature-sensitive; its activity being optimum at 30°C, but only one hundredth at 37°C and not detectable at 40°C (R. Schmitt, unpub. data).

Resolution of cointegrates depends on the presence of 2 res sites in 1 replicon having the same orientation. We have sequenced

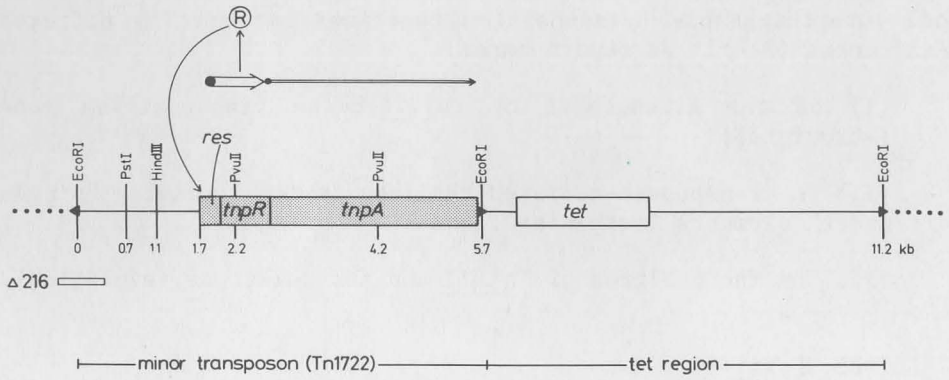


Fig. 1. Diagram of Tn1721 showing central and terminal 38-bp IR (◀▶) flanking the minor transposon (Tn1722) and the repetitious tet region (tet: tetracycline resistance determinant). The homology with Tn501 (shaded) and the location of transposition genes (res: resolution site; tnpR: resolvase; tnpA: transposase), the extent of deletion Δ216 used to construct Tn1722Δ1K (13), relevant restriction sites and their coordinates are indicated. R denotes TnpR protein acting at res (9).

and mapped the crossover site (site I) within a stretch of 11 base pairs, 161-172 bp upstream of the tnpR gene (10). This crossover sequence bears almost no resemblance to the crossover site of Tn3. Footprinting experiments using purified resolvase suggest that there are 2 additional binding sites for resolvase located between the 3' end of tnpR and the crossover point that overlaps the -35 (site II) and -10 boxes (site III) of the tnpR promoter.

"SINGLE-ENDED" TRANSPOSITION

Transposons are characterized by inverted repeats at their ends shown to be essential for transposase-catalyzed transposition (11,12). We have recently reported that truncated derivatives of Tn1721 and Tn21 with a deletion of 1 IR ("single-ended") are capable of mediating transposition of resistance markers to target replicons, if an active tnpA gene (transposase) is present in cis or in trans (13,14). This process has a number of features in common with conventional transposition:

(i) it requires transposase;

(ii) it requires 1 intact IR;

(iii) insertions of donor into target DNA occur at random sites with a strong bias towards AT-rich sequences;

(iv) insertions generate 5-bp direct repeats of target DNA.

Restriction mapping and sequence analyses suggested to us that the resulting fusions between donor and target replicons may be generated by an asymmetric mode of replicative transposition. Accordingly, transposition:

(i) initiates at the intact repeat;

(ii) progresses by replication of the element and the donor vector; and

(iii) terminates at or near the duplicated single IR.

We have analyzed 15 random insertions of Tn1722Δ1K, a "single-ended" derivative of Tn1721 into R plasmid RSF1010 (Fig. 2A). It was found that the element mediates fusions of the donor (pJOE100::Tn1722Δ1K) and target (RSF1010) replicons; the fused portions are nonpermuted (13). In each case, the 2 replicons were joined at the single IR. The second junction was located at or somewhat beyond the duplicated IR in 11 products (class A); 4 fusions ended at various sites within the donor plasmid (class B). Possibly, the latter arose by secondary deletions of class A fusions. The insertions are flanked by a 6-bp direct repeat in 1 and by 5-bp direct repeats of target DNA in 2 independent class A recombinants that have been se

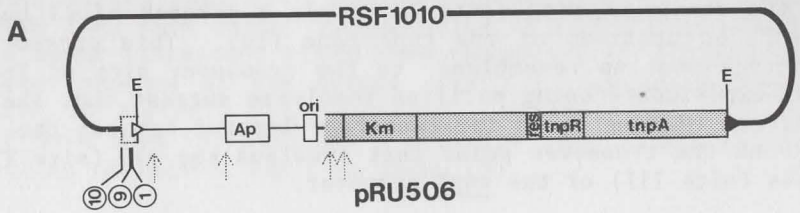


Fig. 2. A Replicon fusion of pRU506 (pJOE100::Tn1722Δ1K; Ref. 7) and RSF1010 mediated by the "single-ended" element Tn1722Δ1K (13). The latter is indicated by a shaded box; the intact IR (▶) and the duplicated IR (▷) plus their EcoRI sites (E), and relevant gene loci are shown (Ap: ampicillin resistance: Km: kanamycin resistance; ori: origin of replication). All fused replicons are joined at the intact IR; the locations of the second junction in 7 independent fusions are marked by dotted arrows and numbered lines, respectively.

B Sequencing from the EcoRI sites across the right-hand and left-hand junctions of 3 fusion products, marked by circled numbers in Fig. 2A. Normal and boldface lettering distinguish between donor and target DNA, respectively; 5-bp (and 6-bp) direct repeats are boxed and lines above sequences indicate the respective portions of intact IR (arrowhead) and duplicated IR (arrowtail; see Fig. 2A). The number of nucleotides of donor DNA extending beyond the duplicated IR is indicated in each sequence.

quenced so far. The target sites are highly AT-rich, a feature also observed in transposition of the wild-type element Tn1721 (R. Schmitt, unpub. data).

It is therefore reasonable to assume that replicon fusion mediated by transposase and a single IR reflects a process operative in normal transposition. The results are compatible with an asymmetric mode of transposition as proposed by Harshey and Bukhari (15) and Galas and Chandler (16). A model involving rolling circle-type replication is shown in Fig. 3. It assumes:

(i) initiation by nick-ligation at the intact repeat;

(ii) progression of the replication fork along the donor transposon; and

(iii) termination at a signal, ideally an intact IR, which, however, may be replaced by secondary signals like a direct repetition of the original IR sequence.

The latter sequences are always less efficient; they are thought to trigger termination by slowing down the progression of the enzyme complex mediating replicative transposition until it stops (imprecisely) at or beyond the duplicated IR. Replicon fusion is then completed by a second nick (involving the same strand that was initially cut) and subsequent ligation to the target strand. This process formally corresponds to direct transposition as opposed to cointegrate formation that requires cutting of the other strand (16). We suggest that cutting of the same strand is a consequence of the fact that the initiating and terminating IR sequences have the same orientation in "single-ended" transposition.

EVOLUTION

Tn21, Tn501, and Tn1721 belong to a subgroup of transposons more closely related to each other than to Tn3 (3). They are closely related by their transposition functions encoded by a 4-kb region of homology and by the sequence of their 38-bp terminal repeats, but they also specify different resistance determinants. Tn21 is 20 kb in size and specifies resistance to mercuric ions (Hg), streptomycin/spectinomycin (Sm/Sp), and sulfonamides (Su) (17). Tn501 is 8.2 kb and specifies Hg (18), and Tn1721 is 11.2 kb and specifies Tc (5). The structures of these elements are compared in Fig. 4.

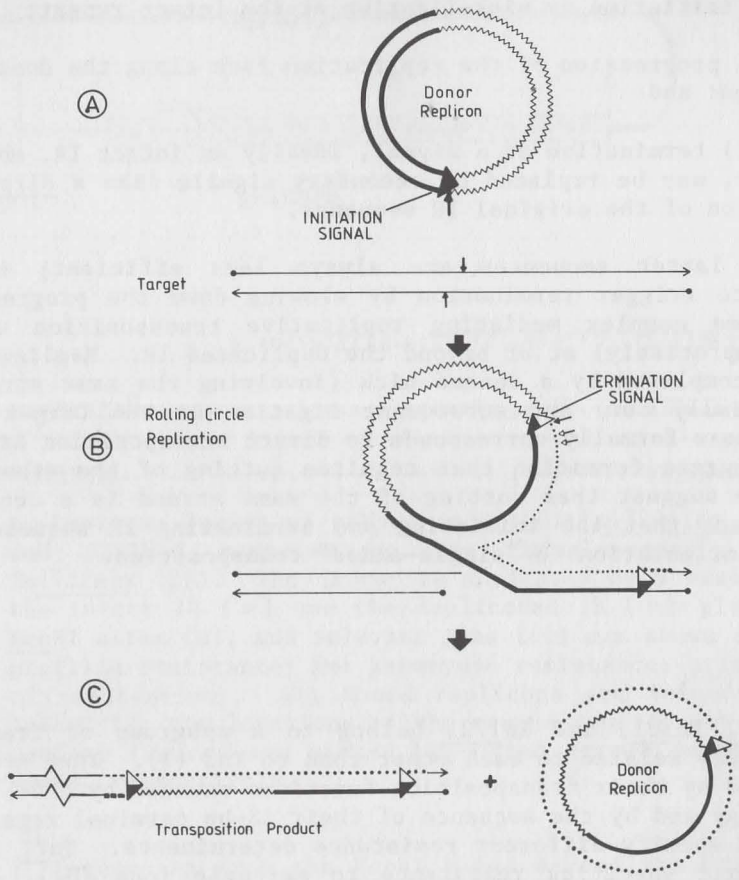
The close structural similarities in this group of elements suggests that their transposition functions have descended from a common ancestral transposable element. The following discussion of transposon evolution will consider 2 aspects:

(i) the possible origin of such an ancestral element and

(ii) modes, by which other genes are acquired to generate the transposable elements that are seen today.

Origin of Transposable Elements

An essential feature of a transposable system is a protein (transposase) that recognizes a specific site (IR) and produces a specific nick at the site acting as an origin for replication. The occurrence of a second copy of the recognition sequence (IR) by dup-



lication would generate a "basic transposon" composed of a tnpA gene flanked by copies of the IR.

The specific recombination system involved in transposition of Tn3-like elements is specified by tnpR/res. The tnpR genes that have been examined show similarities to genes that code for proteins involved in inversion of segments of DNA (4,9) such as the hin gene contained by an invertible element that controls phase variation in Salmonella (19). Such an element may have been incorporated into the "basic transposon." Initially, the segment of DNA retained its invertibility, i.e., it had a recognition site on each side. Deleting 1 of these flanking sites would lock the inversion gene into one orientation or the other, with respect to the tnpA gene. This explanation is in accord with the fact that the orientation of tnpR and res with respect to tnpA in Tn3 is opposite to that in Tn21, Tn501, and Tn1721 (Ref. 4,9; Fig. 4). The ancestral transposable element thus generated consists of (38-bp) inverted repeats flanking a central region that contains tnpA, tnpR, and res. Present day paradigms of such elements are γδ (Tn3 branch) and Tn1722 (Tn21-Tn501-Tn1721 branch).

Fig. 3. Model of asymmetric replicative transposition (15,16) adapted to "single-ended" replicon fusion (13,14).

A Transposition is initiated by nick-ligation at the single intact repeat (▶) and by 5-bp staggered nicks at the target site.

B Nick-ligation of single donor and target DNA strands and rolling circle type replication proceed along the transposable element (——) and the donor vector (〰〰), until a termination signal [duplicated IR (▶) is encountered].

C Cutting of the same single donor DNA strand (arrows) and ligation to free end of target DNA. Nick-ligation involving the same strand bypasses cointegrate formation, unlike in the transposition of "2-ended" elements, where different DNA strands are nicked at the initiation and termination steps, respectively (16). We suggest that the orientation of the terminating IR sequence, whether direct or inverted, is responsible for directing the endonucleolytic activity (of transposase).

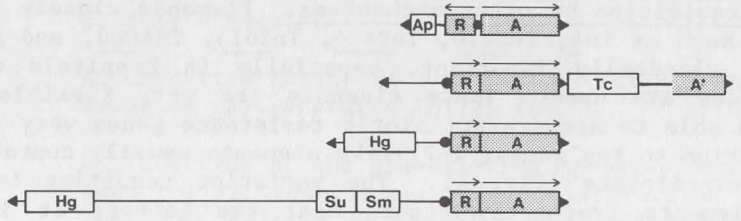


Fig. 4. Structure of Tn3-like transposons superimposed relative to their transposition genes (shaded). All members have related 38-bp ends (◀ ▶), resolvase (R), and transposase (A; A' = truncated). Their transcriptional polarities are indicated by arrows; res is marked by a dot (●). Approximate locations of resistance determinants are shown (resistances to ampicillin, Ap; mercuric ion, Hg; streptomycin, Sm; sulfonamide, Su; tetracycline, Tc).

Acquisition of Other Genes by Transposable Elements

The ancestral transposable element discussed above has acquired various other genes, such as antibiotic- and heavy metal-resistance determinants, during evolution. It is this acquisition that is the most immediately important, and which can be discussed with reasonable confidence. We identify 3 major ways in which the genes can be picked up. In general, subsequent deletions of various parts of the new elements are necessary to generate present day transposons.

(i) Acquisition by concerted action of 2 elements. The structure of Tn1721 originally suggested a general way in which transposons might pick up the DNA sequences (20). Thus, Tn1721 consists of a complete copy of a transposable element (Tn1722) and also a 1.8-kb fragment of the same element with the Tc determinant interspersed (see above). This suggests that, originally, 2 complete copies of a Tn1722-like progenitor inserted on each side of the Tc determinant (still present in RP4; Ref. 21) and that subsequent partial deletion of 1 element resulted in the structure of Tn1721. It is not known yet if deletion formation in this case involved specific sites.

(ii) Acquisition by transposition. Insertion of a pre-existing transposable element into another frequently results in the acquisition of new resistance genes by the latter. A good example is Tn4 (22,23). Here, Tn3 (Ap) is inserted into the Hg^r determinant of an element almost identical with Tn21. Thus, Tn4 is simply Tn21 with an intact Tn3 element in it. This type of reaction has also been implicated in the generation of Tn501 (24). This element contains 1 entire end of Tn2613 identical with this end of Tn21 (25). Thus, the resistance determinant of Tn501 originates from Tn2613. The latter or an element very like it is inserted into a progenitor of the Tn1722-type 80 bp from the end; a subsequent resolvase-catalyzed deletion generated Tn501 (Fig. 5B).

(iii) Acquisition by other mechanisms. Elements closely related to Tn21 such as Tn4, Tn2410, Tn2424, Tn2613, Tn4000, and BP201 (25,26) are clinically important, especially in hospitals where aminoglycosides are used. These elements are very flexible and appear to be able to acquire antibiotic resistance genes very easily. In addition to tnp genes, Tn21-like elements usually contain Hg and Su/Sm determinants (Fig. 4). The variation resulting in the different elements frequently occurs near the latter; it is as though there is a "hot spot" into which other genes can be incorporated (26). Compared with Tn21, various elements differ by insertion of segments of DNA into 2 discrete regions on each side of the aadA gene (Sm). The latter may once have inserted into a single "hot spot," such that it is now divided into 2. The mode of integration into these sites may be by homologous recombination (F. Schmidt, pers. comm.).

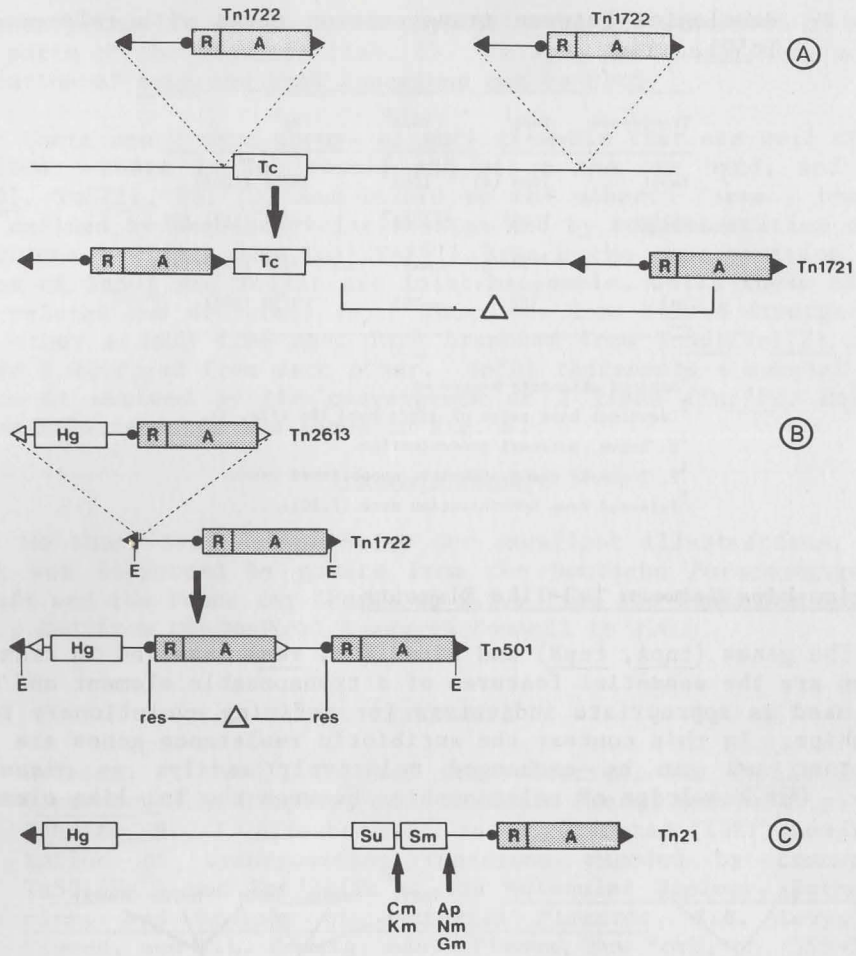


Fig. 5. Modes of acquisition of resistance determinants by transposable elements.

A Concerted action of 2 elements (Tn1722) and subsequent deletion (Δ) generates Tn1721 (6).

B Insertion of the pre-existing Hg transposon Tn2613 (25) into Tn1722; resolvase-catalyzed interaction of the 2 res loci (●) deletes intervening DNA and results in Tn501 (10,24).

C Insertion of various resistance determinants (chloramphenicol, Cm; neomycin, Nm; gentamycin, Gm) into "hot spots" flanking the Sm determinant of Tn21 (26).

Tab. 1. Homologies between transposition genes with reference to Tn501.

Transposon	TnpR[1]	TnpA[1]	IR_R[2]
Tn501	100% (4)	100%[3]	38/38 (100%)
Tn1721	100%[4]	≤100%[5]	36/38 (95%) (7)
Tn21	79% (4)	n.d.	27/38 (71%) (3)
Tn3	32%	30%	19/38 (50%)

[1] Deduced aminoacid sequences.
[2] Identical base pairs of right-hand IRs (Fig. 4).
[3] N. Brown, personal communication.
[4] P. Rogowsky and R. Schmitt, unpublished data.
[5] Inferred from hybridization data (2,20).

Relationships Between Tn3-like Elements

The genes (tnpA, tnpR) and sites (IR, res) involved in transposition are the essential features of a transposable element and were thus used as appropriate indicators for defining evolutionary relationships. In this context the antibiotic resistance genes are less important and can be exchanged relatively easily, as discussed above. Our knowledge of relationships between the Tn3-like elements

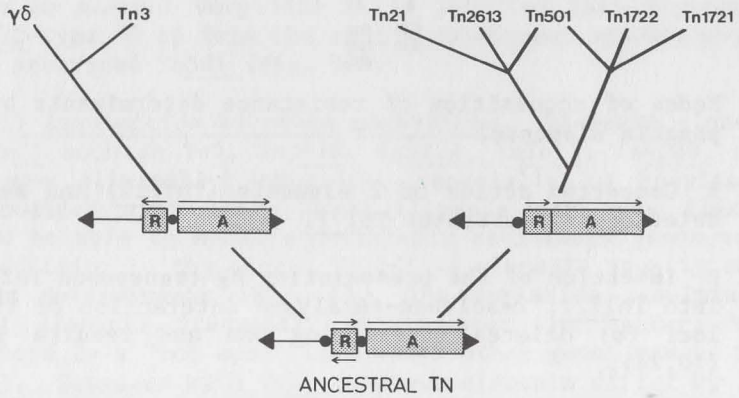


Fig. 6. Pedigree of the Tn3 family derived from restriction, sequence, and complementation data. The 2 main branches diverged early by locking the tnpR-res segment in one or the other orientation relative to tnpA. In each line, diversification occurred by the acquisition of various resistance genes as illustrated in Fig. 5.

depends primarily on the known physical maps and sequences of various parts of the elements (Tab. 1). In some instances, too, complementation of tnpA and tnpR functions can be used.

There are 2 main groups of such elements that are well characterized: there is Tn3 itself and γδ on the one hand, and Tn21, Tn501, Tn1721, Tn1722, and Tn2613 on the other. These 2 branches are defined by sequence relationships and by complementation of tnp functions. Within the Tn21/Tn1721 branch the transposition functions of Tn501 and Tn1721 are interchangeable, while those of Tn21 are related but different (3). Thus, the line Tn3/γδ diverged from the other a long time ago; Tn21 branched from Tn501/Tn1721 before these 2 diverged from each other. Tn501 represents a special case, since it evolved by the convergence of 2 lines (Tn1722, Tn2613). These relationships are shown in Fig. 6.

ACKNOWLEDGMENTS

We thank Mrs. Helene Beier for excellent illustrations. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemie to R.S., from the Fundación March to F.C., and from the Medical Research Council to J.G.

REFERENCES

1. Heffron, F. (1983) Tn3 and its relatives. In Mobile Genetic Elements, J.A. Shapiro, ed. Academic Press, New York. p. 223.
2. Schmitt, R., J. Altenbuchner, and J. Grinsted (1981) Complementation of transposition functions encoded by transposons Tn501(Hg^R) and Tn1721(Tc^R). In Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, S.B. Levy, R.C. Clowes, and E.L. Koenig, eds. Plenum, New York, pp. 359-370.
3. Grinsted, J., F. de la Cruz, J. Altenbuchner, and R. Schmitt (1982) Complementation of transposition of tnpA mutants of Tn3, Tn21, Tn501 and Tn1721. Plasmid 8:276-286.
4. Diver, W.P., J. Grinsted, D.C. Fritzinger, N.L. Brown, J. Altenbuchner, P. Rogowsky, and R. Schmitt (1983) DNA sequences of and complementation by the tnpR genes of Tn21, Tn501 and Tn1721. Mol. Gen. Genet. 181:189-193.
5. Schmitt, R., E. Bernhard, and R. Mattes (1979) Characterisation of Tn1721, a new transposon containing tetracycline genes capable of amplification. Mol. Gen Genet. 172:53-65.
6. Schmitt, R., J. Altenbuchner, K. Wiebauer, W. Arnold, A. Pühler, and F. Schöffl (1981) Basis of transposition and gene amplification by Tn1721 and related tetracycline-resistance transposons. Cold Spring Harbor Symp. Quant. Biol. 45:59-65.
7. Schöffl, F., W. Arnold, A. Pühler, J. Altenbuchner, and R. Schmitt (1981) The tetracycline resistance transposons Tn1721 and Tn1771 have three 38-base pair repeats and generate five-base pair direct repeats. Mol. Gen. Genet. 181:87-94.

8. Wiebauer, K., S. Schraml, S.W. Shales, and R. Schmitt (1981) Tetracylcine resistance transposon Tn1721: recA-dependent gene amplification and expression of tetracycline resistance. J. Bact. 147:851-859.
9. Altenbuchner, J., and R. Schmitt (1983) Transposon Tn1721: Site-specific recombination generates deletions and inversions. Mol. Gen. Genet. 190:300-308.
10. Rogowsky, P., and R. Schmitt (1984) Resolution of a hybrid co-integrate between transposons Tn501 and Tn1721 defines the recombination site. Mol. Gen. Genet. 193:162-166.
11. Heffron, F., B.J. McCarthy, H. Ohtsubo, and E. Ohtsubo (1979) DNA sequence analysis of the transposon Tn3: Three genes and three sites involved in transposition of Tn3. Cell 18:1153-1164.
12. Foster, T.J., M.A. Davis, D.E. Roberts, K. Takeshita, and N. Kleckner (1981) Genetic organization of transposon Tn10. Cell 23:201-213.
13. Mötsch, S., and R. Schmitt (1984) Replicon fusion mediated by a single-ended derivative of transposon Tn1721. Mol. Gen. Genet. (in press).
14. Avila, P., F. de la Cruz, E. Ward, and J. Grinsted (1984) Plasmids containing one inverted repeat of Tn21 can fuse with other plasmids in the presence of Tn21 transposase. Mol. Gen. Genet. (in press).
15. Harshey, R.M., and A.J. Bukhari (1981) A mechanism of DNA transposition. Proc. Natl. Acad. Sci., USA 78:1090-1094.
16. Galas, D.J., and M. Chandler (1981) On the molecular mechanism of transposition. Proc. Natl. Acad. Sci., USA 78:4858-4862.
17. de la Cruz, F., and J. Grinsted (1982) Genetic and molecular characterisation of Tn21, a multiple resistance transposon from R100.1. J. Bact. 151:222-228.
18. Bennett, P.M., J. Grinsted, C.-L. Choi, and M.H. Richmond (1978) Characterization of Tn501, a transposon determining resistance to mercuric ions. Mol. Gen. Genet. 159:101-106.
19. Zieg, J., and M. Simon (1980) Analysis of the nucleotide sequence of an invertible controlling element. Proc. Natl. Acad. Sci., USA 77:4196-4200.
20. Altenbuchner, J., C.-L. Choi, J. Grinsted, R. Schmitt, and M.H. Richmond (1981) The transposons Tn501 (Hg) and Tn1721 (Tc) are related. Genet. Res., Camb. 37:285-289.
21. Waters, S., P. Rogowsky, J. Grinsted, J. Altenbuchner, and R. Schmitt (1983) The tetracycline resistance determinants of RP1 and Tn1721: Nucleotide sequence analysis. Nucl. Acids Res. 11:6089-6105.
22. Kopecko, D.J., J. Bravet, and S.N. Cohen (1976) Involvement of multiple translocating DNA sequences and recombinational hot spots in the structural evolution of bacterial plasmids. J. Mol. Biol. 108:333-360.
23. Foster, T.J., H. Nakahara, A.A. Weiss, and S. Silver (1979) Transposon A-generated mutations in the mercuric resistance genes of plasmid R100.1. J. Bact. 140:176-181.

24. Grinsted, J., and N.L. Brown (1984) Tn501 contains a terminus of Tn21: Consequences for evolution and for complementation. Mol. Gen. Genet. (submitted for publication).
25. Tanaka, M., T. Yamamoto, and T. Sawai (1983) Evolution of complex resistance transposons from an ancestral mercury transposon. J. Bact. 153:1432-1438.
26. Schmidt, F. (1984) The role of insertions, deletions, and substitutions in the evolution of R6 related plasmids encoding aminoglycosides transferase ANT-(2''). Mol. Gen. Genet. (in press).

REPEATED DNA SEQUENCES RECOMBINE 1,000 TIMES MORE FREQUENTLY IN A PLASMID THAN IN THE CHROMOSOME OF BACILLUS SUBTILIS

L. Jannière, B. Niaudet,[1] and S.D. Ehrlich

Institut Jacques Monod, C.N.R.S.
Université Paris VII
2, Place Jussieu
75251 Paris Cedex 05

INTRODUCTION

Escherichia coli chromosome and small ColE1-like plasmids recombine via different pathways. The chromosome follows mainly the RecBC pathway, engages in the RecF pathway only in the absence of exonuclease I and requires the RecA protein to follow the RecE pathway (1,2). The plasmids, on the contrary, recombine predominantly by the RecF and not the RecBC pathway, and engage very efficiently in the RecE pathway (opened by the sbcA mutation) in the absence of the RecA protein (3-6).

Plasmids and chromosomes appear to follow different recombination pathways in Bacillus subtilis as well, as indicated by the fact that (a) 3 out of 12 tested rec genes had different effects on plasmid and chromosome recombination (7); (b) recB and recD mutations inhibited recombination of transforming DNA with the plasmid more than with the chromosome (8); (c) recE mutation had the opposite effect (8-10).

In the present study, we compared recombination frequency between 2 homologous sequences 3.7-3.8 kb long, carried either on a small plasmid or on the chromosome of B. subtilis. We found that the duplication in the plasmid recombined 10^3 times more often than the duplication in the chromosome. The difference was not due to the higher plasmid copy number (about 35 in our study), since the chromosomal sequences tandemly repeated 30 times still recombined

---

[1] Present address: Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15.

much less often than the plasmid sequences. These results support the hypothesis that B. subtilis plasmids and the chromosome recombine in a different way.

APPROACH

To measure recombination frequencies we decided to construct on a B. subtilis genome (plasmid, chromosome) a structure composed of a kanamycin resistance (Km^R) gene flanked by 2 copies of pBR322-derived sequences having the same orientation. Recombination between the repeated sequences should give rise to a circular pBR322-derived molecule carrying the Km^R gene, unable to replicate in B. subtilis and a replicative genome devoid of the Km^R gene. Recombination frequency could then be determined either by following the rate of appearance of the kanamycin-sensitive cells, or by comparing the amounts of the nonreplicative recombinant and the replicative parental Km^R-encoding molecules. The structure needed to measure recombination frequencies was created by a Campbell-like recombination between a transforming circular pBR322 molecule carrying the Km^R gene and a genome (plasmid, chromosome) resident in B. subtilis recipient cells, carrying sequences derived from pBR322. The appropriate resident genomes were constructed and recombined with the transforming plasmid as described below.

CONSTRUCTIONS

The first step involved constructing a plasmid composed of pBR322 and the Km^R gene of pUB110, unable to replicate in B. subtilis. This plasmid was named pHV429 (Fig. 1). The second step consisted of obtaining 2 very similar plasmids, one able to replicate in E. coli and B. subtilis, the other in E. coli only. This was achieved by cleaving the plasmid pHV33 (a hybrid between pBR322 and pC194, Ref. 11) by BamHI, and treating it then with BAL31 and T4 ligase (Fig. 1). A plasmid named pHV33Δ1 able to replicate in E. coli and B. subtilis and a plasmid named pHV33Δ81, able to replicate in E. coli only were thus obtained (another plasmid pHV33Δ11, replicating in both hosts was also obtained). pHV33Δ1, carries 400 bp more of pC194 sequences than pHV33Δ81, part of which is necessary for its replication in B. subtilis (12). The latter plasmid carries about 100 bp more of pBR322 sequences than the former.

To create a structure which could be used to measure recombination frequency in a plasmid we transformed B. subtilis competent cells harboring pHV33Δ1 (or pHV33Δ11) to kanamycin resistance with pHV429. The latter plasmid, which cannot replicate in B. subtilis, must recombine with the homologous sequences (pBR322) of the resident plasmid in order to transform. This recombination gives rise to a plasmid pHV467 (or pHV468, Fig. 1) in which the Km^R gene is flanked by directly repeated pBR322-derived sequences.

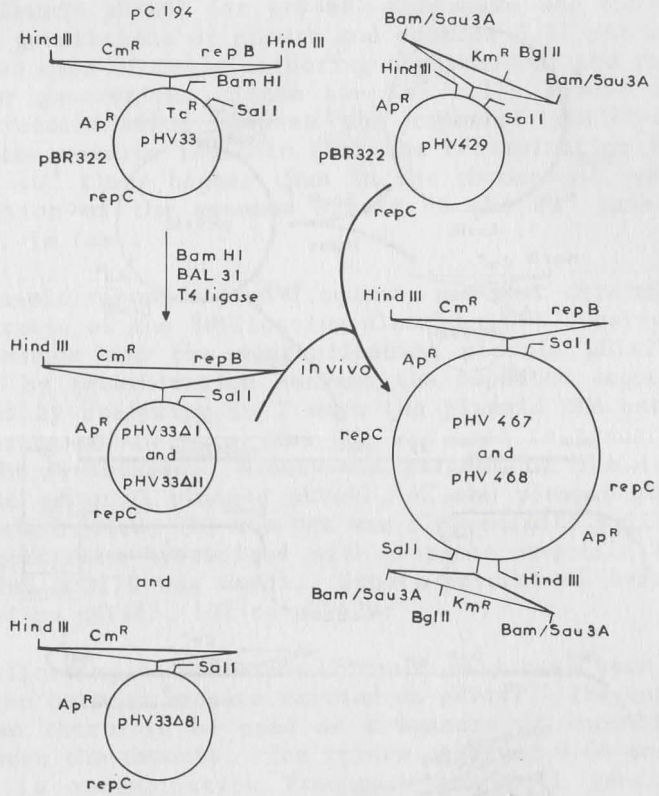


Fig. 1. Construction of plasmids carrying repeated pBR322 sequences.

To create a similar structure in B. subtilis chromosome we followed the procedure outlined in Fig. 2, which consists of 3 steps. First, 2 segments of the B. subtilis chromosome, one carrying the thyB gene, the other of unknown genetic content (labeled X, Fig. 2) were excised from pHV438 (13) and inserted in pHV33Δ81. A plasmid named pHV452 was thus obtained. Second, pHV452 cleaved with BglII was used to transform B. subtilis competent cells to chloramphenicol resistance. This plasmid, unable to replicate in B. subtilis, must recombine with the homologous chromosomal sequences in order to transform. The recombination event replaces wild-type chromosomal sequences between thyB and X with pHV33Δ81 sequences (13) and creates the chromosome named α (Fig. 2). Third, competent cells harboring the α chromosome were transformed to kanamycin resistance with pHV429. Recombination of this plasmid with homologous sequences of the α chromosome (pBR322) gives rise to a chromosome labeled β (Fig. 2) in which the Km^R gene is flanked by directly repeated pBR322 sequences.

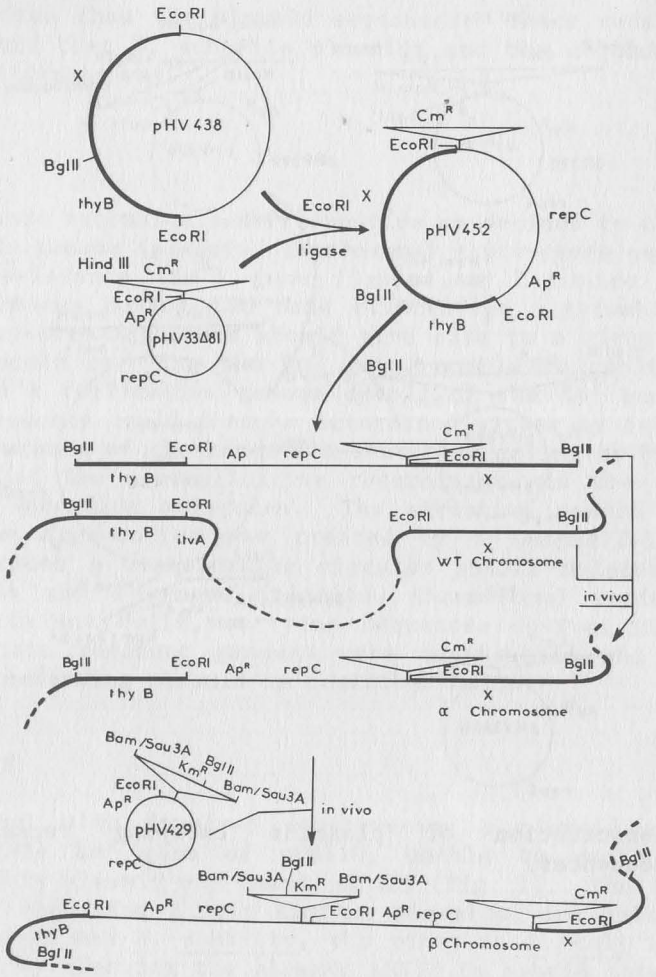


Fig. 2. Construction of a chromosome carrying repeated pBR322 sequences. Thin and thick lines represent sequences of plasmid and chromosome origin; interrupted line, chromosomal sequences replaced by plasmid sequences.

All the constructions made corresponded to those predicted, as judged by analysis of the plasmids (size, restriction patterns, genetic markers) and chromosomes (Southern hybridization, recloning in E. coli of the inserted plasmids and the flanking sequences, and analysis of their size, restriction pattern, and genetic markers).

RECOMBINATION FREQUENCIES

These were first determined by measuring the rate of appearance of cells sensitive to kanamycin (Km^S). In 12 independent clones

harboring plasmid pHV467 (or pHV468) this rate was constant durin at least 60 generations of growth and equaled 0.17 per cell genera tion. In the case of cells harboring β chromosome the rate was onl $1.2.10^{-4}$ per generation. Since the Km^S cells appear as a conse quence of recombination between the repeated pBR322-derived se quences, these results indicate that the recombination frequency i plasmids is 10^3 times higher than in the chromosome, provided tha the segregation of the genomes devoid of the Km^R gene from thos carrying it, is fast.

The plasmid recombination frequency was next determined by mea suring the ratio of the replicating plasmid pHV467, carrying the re peated sequences and the nonreplicating plasmid pHV429, produce from pHV467 by recombination between the repeated sequences. Thi was achieved by analyzing in 2 ways the plasmid DNA extracted fro cells harboring pHV467. (a) The DNA was used to transform E. col to kanamycin resistance. Ninety-six percent of the transformant harbored the parental plasmid pHV467, 4% the plasmid pHV429 gener ated by recombination; (b) the DNA was cleaved with BglII, and afte gel electrophoresis hybridized with a probe specific for the Km gene (labeled pUB110 was used). Ninety percent of hybridizing DN corresponded to pHV467, 10% to pHV429.

Nonreplicating plasmid pHV429 could only have been produced b recombination between repeats carried on pHV467. The ratio of the plasmids can therefore be used as a measure of recombination fre quency between the repeats. The values observed 0.04 and 0.1 shoul correspond to recombination frequency per cell generation. Th difference between the 2 values may be due to integration of newl formed pHV429 into a pHV467 molecule present within the same cell This would give rise to a plasmid having the structure pHV33Δ $(pHV429)_2$, which would not be distinguished from pHV467 (having th structure pHV33Δ1-pHV429, Fig. 1) by our transformation analysis but would give rise to monomeric pHV429 in the restriction/hybridi zation analysis, since only pHV429 carries a BglII site (Fig. 1) Both values were, nevertheless, close to that obtained by followin the rate of appearance of Km^S cells (0.17/generation, see above) which indicates that plasmids devoid of the Km^R gene segregate rapidly from plasmids carrying that gene.

Free pHV429 was not detected by hybridization in the total DN extracted from cells harboring the β chromosome. This indicate that the recombination frequency between the repeated sequence present in the chromosome was below 10^{-3}/generation, the limit o detection of our analysis.

Genetic and biochemical analysis gave values of abou 10^{-1}/generation for the recombination frequency of plasmid-borne se quences. Genetic analysis (the only one having sufficient sensi tivity) gave a value of 10^{-4}/generation for chromosome-borne se quences. Recombination in plasmids is therefore 10^3 times mor frequent than in the chromosome. Could this difference be due t

the presence of a higher number of copies of the plasmids than of the chromosome in the cell? To answer this question, we amplified the repeated structure carried by the chromosome and determined recombination frequency within the amplified structure.

AMPLIFICATION

Gene amplification has been well documented in prokaryotes (14-19). It occurs spontaneously in a number of bacteria provided that they carry in their genome an amplifiable structure of a general form RMR, where R and M stand for different DNA sequences (repeat and marker, respectively). Cells carrying the amplified structure $R(MR)_n$, where n represents the number of repeats of the amplification unit MR, can be isolated when their phenotype differs from that of the parental cells in which amplification did not occur.

The amplifiable structure RMR is present on the β chromosome, where pBR322 sequences and the Km^R gene stand for R and M, respectively (Fig. 2). Cells harboring such a chromosome were resistant to 1 µg/ml of kanamycin. Cells resistant to higher doses of the drug (2-10 µg/ml) appeared with frequencies of 10^{-4}-10^{-6}. They were enriched by growth in appropriate liquid medium, their DNA was extracted and analyzed by restriction enzyme cleavage and gel electrophoresis. Amplification of pBR322 sequences and the Km^R gene (the amplification unit) was detected in all cases, as judged by the appearance of visible restriction bands of the expected size over the background of the chromosomal restriction pattern. The degree of amplification, estimated by densitometry (and in some cases also by hybridization with an appropriate probe) is shown in Tab. 1. It increased with the kanamycin resistance up to 5 µg/ml of antibiotic and then leveled off, probably because of accumulation of mutations rendering the cells less permeable to aminoglycosides. The final value, about 30, is close to that reported for the pC194 copy number, 35 (19a).

RECOMBINATION WITHIN AMPLIFIED STRUCTURE

To measure the recombination frequency within the amplified structure we propagated the cells harboring 30 copies of the amplification unit (which is equivalent to pHV429, Fig. 1) for 145 generations in a medium devoid of antibiotic and (a) determined their plating efficiency on a medium containing 7.5 µg/ml of kanamycin; (b) extracted their DNA and measured the number of copies of the amplification unit.

Plating efficiency of the cells was equal to 1.0. Cells harboring 15 copies of the amplification unit plate on the same medium with an efficiency of 10^{-3}. Assuming that the precision of this

Tab. 1. Amplification of the Km^R gene in B. subtilis cells.

Resistance to kanamycine (µg/ml)	Degree of amplification
1	1
2	9
2.5	15
3	20
4	26
5	30
8	31
10	31

assay was such that we could not distinguish plating efficiency of 0.9 from that of 1.0, the frequency of the loss of 15 copies from the amplified structure could have been 7.10^{-4} per generation.

The copy number of the amplification unit, determined by analysis of DNA extracted from the same cells, was 28, which is probably not significantly different from the starting value of 30. Assuming that the difference was significant, the frequency of the loss of 2 copies of the amplification unit would be 5.10^{-4}/generation.

We also attempted to measure recombination frequency by determining the amount of free nonreplicative plasmid in the cells harboring the amplified structure. For this purpose, DNA from such cells was analyzed by Southern hybridization, using a probe specific for the Km^R gene. No free plasmid was seen under conditions in which about .03 copies/chromosome would have been detected, as judged from a control experiment in which a low amount of pHV429 was deposited on the gel. Recombination frequency was therefore lower than about 10^{-3}/generation.

None of the 3 methods used allowed us to measure with precision the recombination frequency within the chromosomally located amplified structure. The values obtained, ranging from 5.10^{-4} to 10^{-3}/generation are very likely too high, but are nevertheless close to that observed for a single duplication ($1.2.10^{-4}$/generation) and much lower than 10^{-1}/generation observed for plasmids pHV467 and pHV468.

DISCUSSION

Recombination between repeated pBR322 sequences carried on plasmid pC194 occurs 10^3 times more often than between the same se-

quences carried in the thyB region of B. subtilis chromosome. This observation raises several questions.

(A) Do plasmid-borne sequences generally recombine very frequently? It was previously observed that different pUB110-derived repeated sequences, replicated by pUB110 replicator, indeed recombine very frequently (20). In addition, pBR322 sequences carried on pE194 also appear to recombine frequently (unpub. results). Further experiments will, however, be needed to confirm and extend these observations.

(B) Do chromosomally located sequences recombine in general infrequently? We observed no recombination within a structure containing 20 copies of an 11-kb amplification unit, composed of the thyB, X, pBR322, and Km^R sequences, in experiments which should have detected events occurring with a frequency of 7.10^{-4}/generation. On the other hand, repeated and amplified sequences in the pheA (21) and amy (A. Albertini and S. Galizzi, pers. comm.) region of the chromosome recombined with an easily observable frequency. These results suggest that some sequences and/or chromosomal regions may be more prone to recombination than others.

(C) What is the molecular basis for the observed difference in recombination frequency? Several hypotheses may be considered; (i) Duplicated sequences may recombine by an intermolecular rather than intramolecular process, which could possibly occur more frequently between plasmids (22) than between chromosomes. This is ruled out by the fact that the recE4 mutation, which reduces intermolecular plasmid recombination about 100 times (10,23) without affecting intramolecular recombination (24,25) did not change recombination frequency between plasmid-carried repeated sequences studied here; (ii) Recombination stimulating sequences (chi-like elements, Ref. 26) could be carried on plasmids and be absent from the thyB chromosomal region. They should be present not only within 400 bp of pC194 by which autonomously replicating pHV33Δ81 differs from nonreplicating pHV33Δ81 (Fig. 1 and 2), but also on 2 other plasmids, pUB110 and pE194 (see above, A), and should exert an effect on recombination much greater than that (5-8 times), previously described (26,27). We cannot rule out this hypothesis at present, but consider it unlikely; (iii) Recombination stimulating functions, such as oriT of plasmid F (28), may be present on plasmids and not on the chromosome; (iv) The proximity of a replication region may stimulate recombination of repeated sequences. This is supported by the preliminary evidence that initiation of replication in the chromosome induces recombination in its vicinity; (v) Plasmid structure (i.e., degree of supercoiling) may stimulate recombination. Any of the last 3 hypotheses could account for our results and for the fact that plasmids and the chromosome do not follow the same recombination pathways (i.e., do not use with the same efficiencies the enzymes capable of carrying out the process of recombination,

Ref. 1-10). Further experiments will be needed to support one over the others.

It is tempting to speculate about the possible biological significance of the observed difference in recombination rates. Frequent recombination may allow rapid evolution by generating novel genetic assemblies. The cost of this velocity, engendered by the loss of unsuccessful variants, may be acceptable in a polyploid system composed of small genetic units (the energy invested in synthesizing a nonviable copy of a 3-kb plasmid pC194 may not be too high) but intolerable in a predominantly haploid system containing a large genetic unit (the energy invested in creating not only the 4.10^3-kb chromosome of B. subtilis but also the totality of cell components must be very many orders of magnitude higher). Mechanisms ensuring high plasmid and low chromosomal recombination may thus have an evolutionary justification.

Finally, it may be worthwhile to draw attention to the fact that the foreign genetic information is likely to be maintained more stably in an infrequently recombining chromosome than in a frequently recombining plasmid. This consideration may be of importance in devising strategies to construct genetically modified organisms useful to humankind.

ACKNOWLEDGMENTS

We thank P. Noirot and D. Pujante for providing unpublished data and E. Pierre for her excellent technical help. This work was supported, in part, by a grant from the Centre National de la Recherche Scientifique (A.T.P. Biotechnologie).

REFERENCES

1. Fishel, R.A., A.A. James, and R. Kolodner (1981) recA independent general genetic recombination of plasmids. Nature 294:184-186.
2. Laban, D., and A. Cohen (1981) Interplasmidic and intraplasmidic recombination in Escherichia coli K12. Mol. Gen. Genet. 184:200-207.
3. James, A.J., P.T. Morrison, and R. Kolodner (1982) Genetic recombination of bacterial plasmid DNA. Analysis of the effect of recombination deficient mutations on plasmid recombination. J. Mol. Biol. 160:411-430.
4. Potter, M., and D. Dressler, (1979) DNA recombination: In vivo and in vitro studies. Cold Spring Harbor Symp. Quant. Biol. 43:969-985.
5. Clark, A.J. (1973) Recombination deficient mutants of E. coli and other bacteria. Ann. Rev. Genet. 7:67-86.

6. Clark, A.J. (1980) A view of the RecBC and RecF pathways of E. coli recombination. In Mechanistic Studies of DNA Replication and Genetic Recombination, B. Alberts and C.F. Fox, eds. Academic Press, New York, pp. 891-899.
7. Prozorov, A.A., V.I. Bashkirov, N.M. Lakomova, and N.N. Surikov (1982) Study of the phenomenon of marker rescue in plasmid transformation and transduction of intact cells, protoplasts and different rec mutants of Bacillus subtilis. Mol. Gen. Genet. 185:363-364.
8. Weinrauch, Y., and D. Dubnau (1983) Plasmid marker rescue transformation in Bacillus subtilis. J. Bact. 154:1077-1087.
9. Dubnau, D., R. Davidoff-Abelson, B. Scher, and C. Cirigliano (1973) Fate of transforming deoxyribonucleic acid after uptake by competent Bacillus subtilis: Phenotypic characterization of radiation-sensitive recombination-deficient mutants. J. Bact. 114:273-286.
10. Michel, B., B. Niaudet, and S.D. Ehrlich (1983) Intermolecular recombination during transformation of Bacillus subtilis competent cells by monomeric and dimeric plasmids. Plasmid 10:1-10.
11. Primrose, S.B., and S.D. Ehrlich (1981) Isolation of plasmid deletion mutants and study of their instability. Plasmid 6:193-201.
12. Dagert, M., I.M. Jones, A. Goze, S. Romac, B. Niaudet, and S.D. Ehrlich (1984) Replication functions of pC194 are necessary for efficient plasmid transduction by phage M13. EMBO J. 3:81-86.
13. Niaudet, B., A. Goze, and S.D. Ehrlich (1982) Insertional mutagenesis in Bacillus subtilis: Mechanism and use in gene cloning. Gene 19:277-284.
14. Rownd, R.H., and S. Mickel (1971) Dissociation of reassociation of RTF and r determinants of the R-factor NR1 in Proteus mirabilis. Nature New Biology 234:40-43.
15. Anderson, R.D., and J.R. Roth (1977) Tandem genetic duplications in phage and bacteria. Ann. Rev. Microbiol. 31:474-505.
16. Clewell, D.B., V. Yagi, and B. Bauer (1975) Plasmid-determined tetracycline resistance in Streptococcus faecalis: Evidence for gene amplification during growth in presence of tetracycline. Proc. Natl. Acad. Sci., USA 72:1720-1724.
17. Mattes, R., M.J. Burkardt, and R. Schmitt (1979) Repetition of tetracycline resistance determinant genes on R plasmids pRSD1 in Escherichia coli. Mol. Gen. Genet. 168:173-184.
18. Chandler, M., E. Boy de la Tour, D. Williams, and L. Caro (1979) Some properties of the chloramphenicol resistance transposon Tn9. Mol. Gen. Genet. 176:221-231.
19. Peterson, B., and R.H. Rownd (1983) Homologous sequences other than insertion elements can serve as recombination sites in plasmid drug resistance gene amplification. J. Bact. 156:177-185.
19a. Shivakumar, A.G., and D. Dubnau (1978) Plasmid replication in dnats mutants of Bacillus subtilis. Plasmid 1:405-416.
20. Michel, B. (1981) Recombination génétique homologue chez Bacillus subtilis. Thèse 3e cycle à l'Université de Paris VII.

21. Young, M. (1984) Gene amplification in Bacillus subtilis. J. Gen. Microbiol. (in press).
22. Bedbrook, J., and F. Ausubel (1976) Recombination between bacterial plasmids leading to the formation of plasmid multimers. Cell 9:707-716.
23. Keggins, K.M., E.J. Duval, and P.S. Lovett (1978) Recombination between compatible plasmids containing homologous segments requires the Bacillus subtilis recE gene product. J. Bact. 134:514-520.
24. Tanaka, T. (1979) recE4 independent recombination between homologous deoxyribonucleic acid segments of Bacillus subtilis plasmid. J. Bact. 139:775-782.
25. Michel, B., B. Niaudet, and S.D. Ehrlich (1982) Intramolecular recombination during plasmid transformation of Bacillus subtilis competent cells. EMBO J. 1:1505-1511.
26. Stahl, F.W. (1979) Special sites in generalized recombination. Ann Rev. Genet. 13:7-24.
27. James, A.J., P.T. Morrison, and R. Kolodner (1983) Isolation of genetic elements that increase frequencies of plasmid recombination. Nature 303:256-259.
28. Porter, R.D. (1981) Enhanced recombination between F42lac and λplac: dependence on F42lac fertility functions. Mol. Gen. Genet. 184:355-358.

MERCURY RESISTANCE TRANSPOSON Tn*813* MEDIATES CHROMOSOME TRANSFER IN *RHODOPSEUDOMONAS SPHAEROIDES* AND INTERGENERIC TRANSFER OF pBR322

A.R. St. G. Bowen and J.M. Pemberton

Department of Microbiology
University of Queensland
St. Lucia 4067, Australia

INTRODUCTION

Until relatively recent times little was known of the mechanisms of genetic exchange among photosynthetic bacteria (15). Although these organisms have been used as model systems for studying the biochemical and biophysical bases of photosynthesis, photochemical nitrogen fixation, and membrane formation, parallel studies involving genetic analysis have been, at best cursory. Because of the difficulties associated with the growth, mutant isolation and manipulation of obligate anaerobes, facultative photosynthetic bacteria, particularly members of the genus *Rhodopseudomonas*, provided the first detailed insights into mechanisms of genetic exchange among photosynthetic bacteria (14).

After the first discovery by Marrs (11) of genetic exchange in *Rhodopseudomonas capsulata*, mediated by phage-like particles known as Gene Transfer Agents, Sistrom (16) reported chromosome transfer in *Rhodopseudomonas sphaeroides* mediated by the broad host-range plasmid, pR68.45, a plasmid shown to mobilize the chromosomes of a wide variety of gram-negative bacteria (10). Pemberton and Bowen (13) then reported high frequency chromosome transfer in *R. sphaeroides* promoted by the broad host-range IncP-1 plasmid pRP1 carrying the mercury transposon Tn*501*. Using a variety of auxotrophic, antibiotic resistance and photopigment mutations, a map of the genome of *R. sphaeroides* was compiled. The map produced, using pRP1::Tn*501*, consisted of 2 linkage groups. In this paper we compare further mapping data obtained with pRP1::Tn*501* to patterns of marker inheritance from chromosome mobilization promoted by plasmid pR751 carrying a mutant of the mercury transponson Tn*21* deleted for

its resolvase function (Tn813; Ref. 4). These data demonstrate that R. sphaeroides possess but a single linkage group carrying all mutations so far mapped. In addition to mobilizing the chromosome, plasmid pR751::Tn813 is able to mobilize small cloning vectors, such as pBR322, from Escherichia coli to a number of gram-negative bacteria including R. sphaeroides.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Transposons

Details of the bacterial strains, plasmids, and transposons are listed in Tab. 1.

Media and Cultural Conditions

Z broth, Z agar, and minimal medium have been described previously (13). Unless otherwise specified, all cultures were incubated at 35°C.

Antibiotics, Amino Acids, and Mercuric Salts

Because of the sensitivity of certain auxotrophic mutants, particularly phenylalanine mutants, to the levels of amino acids in minimal medium, all amino acids, purines, and pyrimidines were added to a final concentration of 5 µg/ml. For E. coli, the contraselective levels of antibiotics were: carbenicillin, 100 µg/ml; tetracycline hydrochloride, 20 µg/ml; kanamycin sulfate, 50 µg/ml; streptomycin sulfate, 100 µg/ml; mercuric chloride, 12 µg/ml; trimethoprim, 50 µg/ml; rifampicin, 100 µg/ml; naladixic acid, 25 µg/ml. For R. sphaeroides, the antibiotic levels were; carbenicillin, 1 µg/ml; tretracycline hydrochloride, 1 µg/ml; kanamycin sulfate, 20 µg/ml; streptomycin sulfate, 30 µg/ml; mercuric chloride, 7 µg/ml; trimethoprim, 50 µg/ml; rifampicin, 30 µg/ml; naladixic acid, 10 µg/ml.

Isolation of Auxotrophic Mutants and Mating on Solid Medium

These techniques have been described previously (13).

RESULTS

Chromosome Mapping Using RP1::Tn501

Use of RP1::Tn501 produced polarized chromosome transfer in R. sphaeroides, and this discovery has allowed the construction of a genomic map of this bacterium consisting of 2 linkage groups, each possessing early and late markers (13). One of the priorities in extending mapping data obtainable with RP1::Tn501 was to determine

if the large linkage group, containing the photopigment gene cluster, was in fact linked to the small linkage group, containing the histidine gene cluster. Based on the possibility that chromosome transfer was bidirectional from a single origin on a circular R. sphaeroides chromosome, we examined the linkage between later markers of both linkage groups.

The marker furthest from the origin of transfer on the large linkage group was trp-3$^+$ (Fig. 1); with RP1:: Tn501 this marker showed occasional low linkage (1-2%) to his-3$^+$ on the small linkage group. By comparison leu-2$^+$, a late marker on the small linkage group showed more appreciable linkage (8%) to str-14, on the large linkage group. While these low linkage values were inherent in mapping this late region of the chromosome with RP1:: Tn501, there was some hesitation in concluding that the 2 linkage maps could have been unified using mapping data obtained with this plasmid. To provide more substantial linkage values between markers in this late region, a variety of new plasmid-transposon combinations were tested for chromosome mobilizing ability in R. sphaeroides (Bowen and Pemberton, manuscript in preparation). The one chosen for current study was pR751::Tn813.

Chromosome Mobilization and Mapping Using pR751::Tn813

Initial experiments with pR751::Tn813 demonstrated that this plasmid was capable of chromosome mobilization in R. sphaeroides.

A comparison of the level of recovery of recombinants, for markers spanning the entire R. sphaeroides linkage group, suggested that pR751::Tn813 produced chromosome transfer predominantly from an origin close to, and with high frequency transfer of, the photopigment gene cluster (Tab. 2). Using the same group of markers, plasmid pRP1::Tn501 showed its characteristic polarized transfer from origins close to the photopigment gene cluster, and the histidine gene cluster (Tab. 2; Fig. 1; Ref. 13). A linkage analysis of recombinants derived from crosses involving pR751::Tn813 showed substantial coinheritance between markers in the late region (leu-2$^+$, trp-3$^+$, and str-14) of the R. sphaeroides chromosome (Fig. 2). These data confirmed that there is but one linkage group, containing all known markers. The pattern of coinheritance was such as to suggest that pR751::Tn813 transfers large segments of the main chromosome, perhaps occasionally the entire chromosome. Using both pR751::Tn813 and pRP1::Tn501 a map of the R. sphaeroides chromosome was produced (Fig. 3); the relative positions of each of the markers was determined by linkage to surrounding genes.

Intergeneric Transfer of Cloning Vectors

Photosynthesis is but one of many interesting characteristics of gram-negative bacteria, not expressed in E. coli (1). A number of solutions have been devised which allow cloning into genetically

Tab. 1. Bacterial strains, plasmids, and transposons.

Strain Number	Genotype/Phenotype	Reference
Bacterial strains		
Rhodopseudomonas sphaeroides		
RS6122	his-1 1	(13)
RS6124	his-2	(13)
RS6127	arg-1	(13)
RS6132	his-3	(13)
RS6152	ade-3,crtE1,str-14	(13)
RS6167	his-4	(13)
RS6168	phe-1	(13)
RS6169	arg-2	(13)
RS6177	met-2,arg-4	(13)
RS6178	met-2,phe-2	(13)
RS6181	his-3,phe-4	(13)
RS6182	his-3,aro-2	(13)
RS6189	met-2,trp-1,his-6	This paper
RS6192	met-2,his-5,gly-1	(13)
RS6194	his-3,trp-3,phe-5	(13)
RS6195	his-3,trp-3,leu-1	(13)
RS6196	his-3,trp-3,arg-6	This paper
RS6197	his-3,met-5,leu-2	(13)
RS6198	his-3,met-5,phe-6	(13)
RS6199	his-3,met-5,gly-3	This paper
RS6200	his-3,met-5,trp-5	(13)
RS6202	his-3,ile-2,phe-7	(13)
RS6203	his-3,ile-2,gly-4	This paper
RS6204	his-3,aro-1,leu-3	(13)
RS6272	ade-3,crtE1 str-14,rif-10	This paper
RS6304	nif-1	This paper
RS7009	ade-3,crtE1,str-14,rsrI-1,rif-11	This paper
RS7014	his-1,mmc-1	This paper
Escherichia coli		
CSH358	$hsdR^-_k$ $hsdM^+_k$ supF $\phi 80^r$	(5)
JM103	Δ(lac pro),thi,strA,supE, endA,sbcB,$hsdR^-$, F' traD36,proAB, $lacI^q$,ZΔM15	(18)
Plasmids		
pRP1	Tra^+ $Ap^r Km^r Tc^r$	(10)
pR751	Tra^+ $Tp^r Su^r$	(10)
pUC8	Ap^r	(18)
pBR322	$Ap^r Tc^r$	(12)

Tab. 1. Continued

Strain Number	Genotype/Phenotype	Reference
Transposons		
Tn501	$TnpA^+TnpR^+$ Hg^r	(17)
Tn813	$TnpA^+TnpR^-$ Hg^r	(4)

Abbreviations: ade,adenine; Ap^r,ampicillin resistance; aro, aromatic amino acid;arg,arginine;bch,bacteriochlorophyll; crt,carotenoid;end,endonuclease;glyglycine;his,histidine; Hg^r,mercury resistance;hsdM,host-determined modification; hsdR,host determined restriction;ile,isoleucine;Km^r,kanamycin resistance;lac,lactose;leu,leucine;met,methionine; mmc,mitomycin sensitivity; nif,nitrogen fixation;phe,phenylalanine; pro,proline; rif^r,rifampicin resistance; rsrI,restriction endonuclease RsrI; sbc,suppressor for recBC; str,streptomycin resistance;nal^r,naladixic acid resistance; Su^r,sulphonamide resistance; thi,thiamine; Tc^r,tetracycline resistance;Tp^r,trimethoprim resistance;tra,transfer proficiency;trp,tryptophan;Δ,deletion.

well-characterized organisms such as E. coli and subsequent transfer of the cloned genes back into their original host for expression. On the one hand, there are the dual cloning vectors which can replicate and be maintained both in E. coli and the other organism of interest; many such dual cloning vectors have been constructed in vitro by combining pBR322 with a naturally-occurring plasmid isolated from the organism of interest (12).

On the other hand, advantage has been taken of the broad host-range of the small, multi-copy plasmids of the IncQ group; although nonconjugative, plasmids such as pRSF1010, pR300B, and pR1162 can be mobilized by pRP1 to a wide variety of gram-negative bacterial species; Timmis and his coworkers have developed a variety of cloning vectors derived from pRSF1010 by in vitro manipulation (1). Although these 2 approaches have proven useful cloning strategies, the narrow host-range plasmid pBR322 and its many derivatives still remain the most popular cloning vectors available; more recent derivatives, such as pUC8 and pUC12, have the added advantage of being able to be used directly in sequencing by the method of Maxam and Gilbert (18).

In experiments involving the transposon Tn21, Grinsted and his coworkers (4) observed that a mutant deleted for its resolvase function (Tn813) was able to mediate the formation of highly stable cointegrates between 2 plasmids. Using the broad host-range plasmid

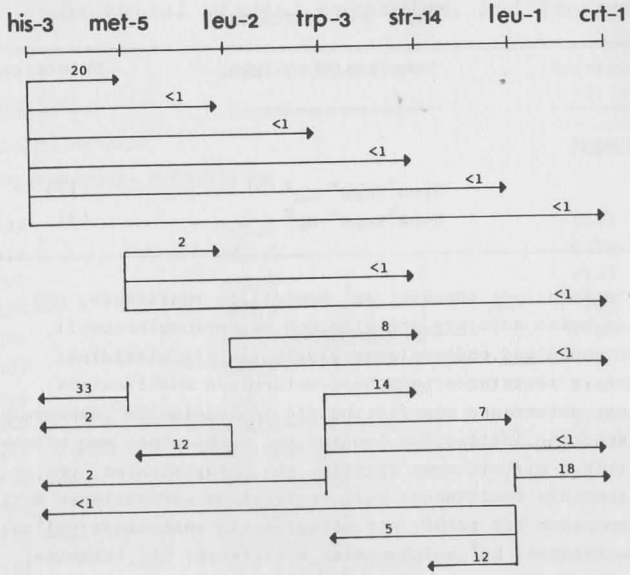


Fig. 1. Linkage data derived from matings between the donor strain RS6152 (pRP1::Tn501) and recipient strains RS6195 and RS6197. Selected marker (bar); unselected marker (arrowhead).

pR751 carrying this mutant transposon, we found pR751::Tn813 formed highly stable cointegrates with pBR322; if the level of cointegrate formation is measured by the level of cotransfer of pBR322 and pR751::Tn813, then approximately 1% of cells harboring both plasmids possess such stable cointegrates. The stability of these cointegrates (pR751::Tn813::pBR322) was such as to allow transfer of pBR322 from one strain of E. coli to another, and from E. coli to R. sphaeroides; the cointegrate containing pBR322 could be retransferred from R. sphaeroides to E. coli (Tab. 3). The presence of the cointegrate in the recipient strain conferred simultaneous resistance to trimethoprim (pR751), mercury (Tn813), carbenicillin, and tetracycline (pBR322). Other small cloning vectors such as pUC8 (Tab. 3), pHC79 (the cosmid derivative of pBR322), and pACYC184 could be transferred between strains of E. coli and from E. coli to R. sphaeroides (Bowen and Pemberton, unpub. data). In addition, the cointegrates containing pBR322 could be transferred from E. coli to such diverse gram-negative bacteria as the cellulolytic Cellvibrio mixtus, the amylolytic Aeromonas punctata, and the xenobiotic degrader Alcaligenes eutrophus (Pemberton, Wynne, and Gobius, unpub. data).

DISCUSSION

Following the discovery that RP1::Tn501 could be used for

Tab. 2. Comparison of the chromosome mobilizing abilities of pRP1::Tn501 and pR751::Tn813.

Selected Marker[b]	Frequency of recombinant formation[a]	
	Plasmids	
Group A	pRP1::Tn501	pR751::Tn813
his-3$^+$	230	16
met-5$^+$	120	64
leu-2$^+$	1.4	60
trp-3$^+$	5.6	30
str-14	0.4	42
leu-1$^+$	5.0	29
phe-5$^+$	6.0	18
Group B		
crt-1$^+$	175	210
ade-3+	190	255

[a] Frequency of recombinants calculated per 10^6 donor cells

[b] In crosses involving Group A markers the donor strain was RS6152 carrying pRP1::Tn501 or pR751::Tn813; for Group B markers, the donor strain was Rs6132 carrying the appropriate plasmid.

genomic mapping in R. sphaeroides, it became possible to locate a wide variety of genes (13). The preliminary map of this bacterium showed 2 linkage groups, with the important photopigment genes clustered together in a single region of the genome close to an origin of chromosome transfer. If it is assumed that R. sphaeroides has a single circular chromosome, then the pattern of recovery of recombinants and the linkage data obtained with RP1::Tn501 is consistent with bidirectional Hfr chromosome mobilization from a single origin on the main chromosome (Ref. 13, 14; Fig. 1; Tab. 2). Confirmation that R. sphaeroides does possess a single linkage group was obtained using pR751::Tn813; in this case, Hfr chromosome mobilization occurs predominantly from a single origin, in one orientation, and with the early transfer of the photopigment genes and the late transfer of the histidine biosynthetic genes (Fig. 1,2; Tab. 2). A contrasting characteristic of pR751::Tn813 compared to pRP:Tn501 is that the former plasmid-transposon combination transfers larger segments of the chromosome (Fig. 1 and 2); the reason for this difference remains unknown.

Evolutionarily related transposons such as Tn3, Tn501, Tn1721, and Tn21 mediate the cointegration of 2 replicons as an intermediate step in their transposition. The cointegrate is then resolved by a

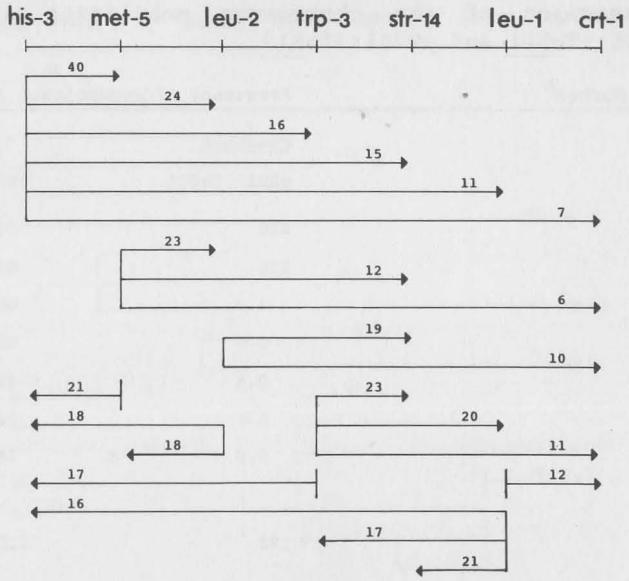


Fig. 2. Linkage data derived from matings between the donor strain RS6152 (pR751::Tn813) and recipient strains RS6195 and RS6197.

site-specific tnpR gene product (resolvase) acting at the res site (4). Usually, stable cointegrates are obtained using transposons carrying either a mutation in the res site or in the tnpR gene. However, Kitts et al. (6) reported that Hg^{++} induces readthrough

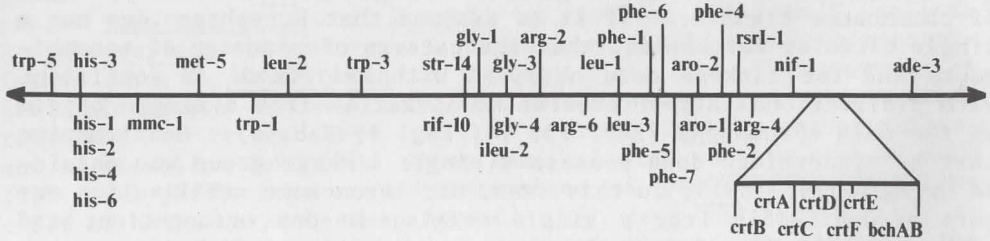


Fig. 3. Linkage map of the Rhodopseudomonas sphaeroides chromosome. The relative positions of markers were determined by patterns of coinheritance with surrounding markers using RP1::Tn501 and pR751::Tn813 promoted chromosome transfer. Gene symbol abbreviations as in Tab. 1. Arrowheads indicate position and orientation of Tn501 transfer origins; Tn813 promotes chromosome transfer from the origin close to ade-3+, with the same orientation as Tn501. The photopigment gene cluster is drawn from data in Ref. 13.

Tab. 3. Transfer of cointegrates between Escherichia coli and Rhodopseudomonas sphaeroides.

Donor	Recipient	Frequency of Transfer[a] Selected markers	
Escherichia coli	Rhodopseudomonas sphaeroides	Tp^rRif^r	$Tp^rCb^rRif^r$
CSH358(pR751::Tn813,pBR322)	RS7009 Res^-Rif^r	$1x10^{-1}$	$1x10^{-3}$
CSH358(pR751::Tn813,pBR322)	RS6152 Res^+Rif^r	$5x10^{-4}$	$1x10^{-6}$
JM103 (pR751::Tn813,pUC8)	RS7009 Res^-Rif^r	$2x10^{-2}$	$5x10^{-5}$
Rhodopseudomonas sphaeroides	Escherichia coli	Tp^rNal^r	$Tp^rCb^rNal^r$
RS7009(pR751::Tn813::pBR322)	CSH358 Res^-Nal^r	$1x10^{-1}$	$1x10^{-3}$
RS7009(pR751::Tn813::pUC8)	CSH358 Res^-Nal^r	$4x10^{-2}$	$5x10^{-4}$

[a]Frequency of transfer calculated per donor cell.

transcription from the mercury-resistance operon into the tnpR gene. These data support the suggestion that the tnpR gene of Tn501 may not have an independent promoter, while tnpA does; this provides an explanation for the efficient transposition and cointegrate formation by Tn501, and the highly inefficient resolution of these cointegrates. The chromosome transfer observed in R. sphaeroides appears to be due to cointegration of RP1::Tn501 and the main chromosome to form an Hfr; more recent reports of Tn501 mediated chromosome transfer in Pseudomonas putida and Bordetella pertussis support this view (3,19). Although Tn3 and Tn1721 do not promote chromosome transfer in R. sphaeroides, this may simply reflect the known efficiency of their resolvase functions, indicating that resolvase-deficient mutants of these 2 transposons could be used for replicon mobilization.

The generation of a linkage map of R. sphaeroides, the availability of a range of mutations in each region of the chromosome, and the ability to transfer small cloning vectors, such as pUC8, pBR322, and its cosmid derivative, pHC79, from E. coli to R. sphaeroides via a cointegrate with pR751::Tn813 provides the opportunity to produce a detailed physical and genetic map of this photosynthetic bacterium. It should be possible to isolate and identify sequences of the chromosome encoding such important biological processes as photosynthesis, photochemical nitrogen fixation, and membrane formation.

ACKNOWLEDGMENTS

The authors wish to thank Cathy Smith and Ann van Pham for their expert technical assistance. A.R.St.G.B. is supported by a Commonwealth Postgraduate Award from the Australian Government. Research funds were provided by the Australian Research Grants Commission, grant number D2/83/155701.

REFERENCES

1. Bagdasarian, M., and K.N. Timmis (1982) Host: Vector systems for gene cloning in Pseudomonas. In Current Topics in Microbiology and Immunology. Springer-Verlag, Berlin, 96:47-67.
2. De La Cruz, F. and J. Grinsted (1982) Genetic and molecular characterization of Tn21, a multiple-resistance transposon from R100-1. J. Bact. 151:222-228.
3. Dean, H.F., and A.F. Morgan (1983) Integration of R91-5::Tn501 into the Pseudomonas putida PPN chromosome and genetic circularity of the chromosomal map. J. Bact. 153:485-497.
4. Diver, W.P., J. Grinsted, D.C. Fritzinger, N.L. Brown, J. Altenbuchner, P. Rogowsky, and R. Schmitt (1983) DNA sequences of and complementation by the tnpR genes of Tn21, Tn501 and Tn1721. Mol. Gen. Genet. 191:189-193.
5. Karn, J., S. Brenner, L. Barnett, and G. Cesarini (1980) Novel bacteriophage cloning vector. Proc. Natl. Acad. Sci., USA 77:5172-5176.
6. Kitts, P., D. Symington, M. Burke, R. Reed, and D. Sherratt (1982) Transposon-specified site-specific recombination. Proc. Natl. Acad. Sci., USA 79:46-50.
7. Gest, H. (1966) Comparative biochemistry of photosynthetic processes. Nature (Lond.) 209:879-882.
8. Grinsted, J., F. De La Cruz, J. Altenbuchner, and R. Schmitt (1982) Complementation of transposition of tnpA mutants of Tn3, Tn21, Tn501 and Tn1721. Plasmid 8:276-286.
9. Hohn, B., and J. Collins (1980) A small cosmid for efficient cloning of large DNA fragments. Gene 11:291-298.
10. Holloway, B.W. (1978) Plasmids that mobilise the bacterial chromosome. Plasmid 2:1-19.
11. Marrs, B.L. (1974) Genetic recombination in Rhodopseudomonas capsulata. Proc. Natl. Acad. Sci., USA 71:971-974.
12. Old, R.W., and S.B. Primrose (1982) Principles of gene manipulation: An introduction to genetic engineering. Blackwell Scientific Publications (Lond).
13. Pemberton, J.M., and A.R.St.G. Bowen (1982) High frequency chromosome transfer in Rhodopseudomonas sphaeroides promoted by the broad host range plasmid RP1 carrying the mercury transposon Tn 501. J. Bact. 147:110-117.
14. Pemberton, J.M., S. Cooke, and A.R.St.G. Bowen (1983) Gene transfer mechanisms among members of the genus Rhodopseudomonas. Ann. Microbiol. (Inst. Pasteur) 134B:195-204.

15. Saunders, V.A. (1982) Genetics and molecular biology of photosynthetic bacteria and cyanobacteria. In Photosynthesis: Development, Carbon Metabolism and Plant Productivity Vol. III. Academic Press, Inc., New York.
16. Sistrom, W.R. (1977) Transfer of chromosomal genes mediated by plasmid R68.45 in Rhodopseudomonas sphaeroides. J. Bact. 131:526-532.
17. Stanisich, V.A., P.M. Bennett, and M.H. Richmond (1977) Characterisation of a translocation unit encoding resistance to mercuric ions that occurs on a non-conjugative plasmid in Pseudomonas aeruginosa. J. Bact. 129:1227-1233.
18. Vieira, J., and J. Messing (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.
19. Weiss, A.A., and S. Falkow (1983) Transposon insertion and subsequent donor formation promoted by Tn501 in Bordetella pertussis. J. Bact. 153:304-309.

REPORT ON A WORKSHOP:

PLASMIDS IN UNUSUAL SYSTEMS

Simon Silver, John Pemberton[1],
Kenji Sakaguchi[2]

Washington University
St. Louis, Missouri, 63110

The first problem for this workshop was to define "unusual systems" as more than a catch-all for topics not covered elsewhere at the meeting. This is related to the "specialized functions" sessions held later in the week, for functions not covered in special single topic sessions. Unusual systems are (i) plasmids in organisms other than E. coli and especially those that have not gained sufficient research activity to stand alone at the meeting. Thus, some "unusual systems" of 1984 will enjoy a different status in future Plasmid Symposia. (ii) Unusual systems also mean plasmid functions, and especially metabolic capacities, governed by plasmids that have not yet seen sufficient study to stand in sections by themselves.

An emerging trend in prokaryotic molecular genetics is the increasing interest in unusual microorganisms; "unusual" meaning organisms which possess biologically important properties (for example, photosynthesis) but for which there are few, if any, means of genetic analysis. There are a number of factors which are stimulating this interest. On the one hand, there is the increasing involvement and influence of biotechnology/genetic engineering companies which are attempting to obtain microorganisms with novel biological properties and which are examining ways of increasing the efficiency of known important medical, industrial, and agricultural processes. On the other hand, there is an increasing awareness by microbial geneticists that it is possible to blend and extend the range of genetic and molecular biological techniques developed in E. coli to many interesting and important species of both gram-positive and gram-negative bacteria.

---

[1]Dept. of Microbiology, University of Queensland, St. Lucia 4067, Australia. [2]Mitsubishi-Kasei Institute of Life Sciences, Minamiooya 11, Machida, Tokyo, Japan, 194

The discussion included how systems of genetic exchange in well-known organisms such as E. coli and P. aeruginosa might be adapted for genetically less well characterized bacteria such as Nitrobacter, Bacteroides, Clostridium and Methanobacterium. For many of these organisms, the broad host-range plasmids and cloning vectors, used so successfully in members of the genus Pseudomonas, are either unstable or simply not transferrable. Another hurdle to overcome is the problem of strain improvement when a biologically important property is encoded in a large number of genes. A common strategy is to carry out cloning in E. coli and transfer the genes back into the bacterium of interest for expression and genetic analysis. This latter approach necessitates more than just a rudimentary understanding of the genetic topography of the unusual bacterium. Questions of gene transfer systems in Xanthomonas (for xantham gum production), Bacteroides nodosus (for production of antipilus vaccine against footrot), Nitrobacter (conversion of nitrite to nitrate), Thermus (heat-stable proteins and biological processes) and Clostridium acetobutylicium (industrial chemicals) were a few of those organisms discussed. Possibilities of setting up systems of conjugation and chromosome transfer (considered the most potentially useful), and transformation and transduction were canvassed. The most popular approach was to be cloning in E. coli and adapting naturally occurring plasmids as potential "shuttle" vectors. One major impediment was a lack of awareness of the range of cloning vectors and strategies that are available for developing gene transfer systems in unusual bacteria.

In addition to the interest in organisms and what they can do, there was an interest in the metabolic products and capacities that are "native" to the organisms and also in products that can be produced by genetic systems cloned into the organism of choice. Among the industrially useful products discussed were amino acids and heat-stable enzymes (as biocatalysts and marketable products). Solvent production might be improved by use of genetically altered organisms with greater solvent resistance. Calf renin and chymosin are bulk products that will be manufactured more economically from bacterial cells rather than isolated from animal tissues. Higher value, smaller market products include peptide hormones.

There was a ready exchange during the workshop and elsewhere during the meeting that showed that people functioning in university laboratories were greatly concerned with producing plasmids and strains with important economic values and, equally, that people working in biotechnology companies were interested in the basic scientific understanding of the systems being developed. It was considered that basic understanding was essential for development of useful strains and their maintenance and improvement.

CHAIRMAN'S INTRODUCTION:

REPLICATION, INCOMPATIBILITY, AND PARTITION

Kurt Nordström

Department of Microbiology
Biomedical Center
Uppsala University
Box 581
S-751 23 Uppsala, Sweden

The life cycle of plasmids comprises 2 different events, increase in number (replication) and distribution of the plasmid copies to the daughter cells (partition). Both these events are controlled by plasmid-borne genetic information. They were discussed in 16 talks, one informal workshop (on partition) and 37 posters.

Replication and partition are independent processes. Deletion of the par region of a plasmid does not affect the control characteristics of plasmid replication but causes unstable inheritance of the plasmid. A par-deleted replicon can be stabilized by the par region of any plasmid. Hence, it is possible to study replication and partition independently.

Replication

Plasmid replication is controlled such that a given plasmid in a given host under given conditions is present in a defined average number of copies per cell. Theoretically, this could be achieved by saturation kinetics where replication is controlled by a rate-limiting host-controlled reaction (Nordström). However, all plasmids studied so far control their own replication by genetic information causing negative control. The main inhibitor can either be protein (λdv, Matsubara), RNA (ColDF13, Nijkamp; pT181, Novick; ColE1, Polisky; FII plasmids, Rownd) or series if direct repeat [R1K2, Figurski; R6K, Helinski; P1, Yarmolinsky; and presumably R116 (RSF1010), Meyer; pSC101, Churchward et al.]. The functioning of the λdv system is fairly well understood, although the biochemical events at initiation of replication still are poorly known

Matsubara addressed the question as to where the first deoxyribonucleotide is introduced during initiation of replication of λdv (Tsurimoto et al.). Similarly, 3 replication control systems using small, unstable RNA species as replication inhibitors are fairly well understood. In all 3 (ColE1-CloDF13, Polisky, Nijkamp; FII plasmids, Rownd; pT181, Novick), the inhibitor has a high degree of secondary structure and interacts with the complementary region of the opposite long transcript. This presumably results in an RNA duplex (Tamm et al.), that changes the configuration of the longer transcript further downstream. This results in a decreased efficiency of primer activity (ColE1-CloDF13) or of expression of a rate-limiting Rep protein (FII and pT181); Wong et al. reported ColE1 mutations changing the secondary structure of the preprimer and affecting replication control. The hypothetical structure of RNAI (the inhibitor of plasmid ColE1) was verified by structural studies presented by Polisky. Similarly, Rownd presented computer studies showing that the secondary structure of the RepA-message of FII plasmids may change by the formation of an RNA duplex with CopA-RNA such that the Shine-Dalgarno region becomes hidden in a duplex region. This may explain why the inhibitory CopA-RNA acts as a translational inhibitor. Interestingly, the Rep protein of FII plasmids is mainly cis-acting, whereas that of plasmid pT181 is also acting in trans. This has some consequences for the incompatibility properties as discussed by Novick.

The large group of important plasmids that have series of DNA repeats as their replication inhibitors was discussed in several papers. As pointed out by Helinski, there are many similarities between the structure of these replicons: the size and spacing of the repeats, the location of at least some of the repeats in the vicinity of the origin, the size of the plasmid-coded Rep proteins (28-38 kb), the location of the rep genes near the origin, the autoregulation of the expression of the rep genes. The function of the repeats is still obscure, but titration of a positively needed function has been suggested, but has not yet been proven. The plasmid-coded proteins are naturally the prime suspects and Yarmolinsky reported that the RepA protein of plasmid P1 binds to the incA repeats. High concentrations of the R6K Rep protein (π) and the P1 Rep protein (RepA) actually inhibit replication (Helinski; Yarmolinsky). Titration of an autoregulated Rep protein maybe poses a paradox. Yarmolinsky tried to solve this paradox with his beautiful Clepsydra (water clock) analogy. The solution could be a trifunctional Rep protein (autoregulated, titrated, and active) as suggested by Kline and Trawick. Results reported by Yarmolinsky support this picture; the RepA protein inhibits transcription from and into the origin of plasmid P1. Furthermore, the P1 replication system works properly even if the origin is physically separated from the incA repeats and the autoregulated repA gene. Hence, the signal caused by binding of the RepA protein to the repeats has to travel through the cytoplasm.

Plasmid RK2 has got an even more complicated control system than the other plasmids with inc repeats. Two proteins, the korA and korB products, interfere with the expression of the Rep protein coded for by the trfA gene (Figurski; Thomas et al.).

A considerable fraction of the contributions dealt with the genetic organization of the various basic replicons. Hence, the knowledge about the structure of the replicons is advancing fast. Similarly, knowledge of the molecular biology is also expanding rapidly. Particularly the ColE1-CloDF13 systems are exploited in many ways: Dooley et al. aim at a total characterization of the replication control region of plasmid ColE1 by systematically mutating "every single base pair"; Elble et al. have isolated copy mutants of ColE1 where the stability of RNAI is changed; van Putten et al. reported that methylation in the control region is important for copy number control of plasmid CloDF13; Naito et al. reported that RNaseH does not seem to be needed for in vivo replication of plasmid ColE1 in contrast to in vitro results reported previously.

The biochemistry of replication is studied intensely in several laboratories. Koguni described a purified system for replication of oriC plasmids of Escherichia coli that fulfills all characteristics of the in vivo system. Similarly Haring et al. and Scholz et al. reported in vitro studies of replication of plasmid RSF1010. This plasmid codes for 3 rep genes and is independent of several of the E. coli DNA replication enzymes (e.g., RNA polymerase and the DnaB, C, and G proteins). This implies a unique replication system which may be the key to the broad host range expressed by plasmid RSF1010. The host range of plasmid RK2 requires specific plasmid-coded functions in some hosts but not in others (Schmidhauser and Helinski).

Less progress has been made in the physiological aspects of plasmid replication. Highlander and Novick studied replication of plasmid pT181 during copy-number-shift experiments. Rownd discussed measurements of the concentration of the various transcripts of the basic replicon of the FII plasmid NR1 at different concentrations of the corresponding genes. Furthermore, Rownd has performed an interesting theoretical analysis based upon the thermodynamics of the interaction between the loops of the CopA-RNA and the RepA-message. These measurements and calculations came to the same conclusions as the physiological experiments performed in other laboratories during the last 10 years, which is reassuring.

Finally, Pritchard discussed the differences between control of plasmid replication and chromosome replication. Perhaps the chromosome uses other principles than the plasmids do? It is striking that oriC plasmids do not express inhibitor activity or sensitivity to a possible inhibitor of replication. There are also differences with respect to the timing of chromosome and plasmid replication.

The picture that now emerges is one in which different plasmids use different approaches to control their replication - there are at least 3-4 different schemes known so far. The idea that plasmids are simple-minded organisms may have received a blow!

Partition

Most naturally occurring plasmids are maintained very stably in exponentially growing populations of host bacteria. However, deletion of regions of plasmids often causes destabilization of the plasmids. Deletion derivatives of low copy-number plasmids are often found to be lost in a frequency that fits with random distribution of the plasmid copies at cell division (P1, Austin; F, Hiraga). However, random distribution of a high copy-number plasmid would not cause any instability of inheritance. Kasner and Rownd reduced the copy number of plasmid ColE1 and observed unstable maintenance and concluded, as others have before, that ColE1 does not have any *par* function. However, deletion derivatives of high copy-number plasmids are often lost in a high frequency (pSC101, Cohen; *oriC*, Kunze and Messer), whereas the parent plasmids are stably maintained. This implies that under these circumstances, the unit of segregation consists of more than one plasmid genome, either by the formation of concatemers or by some sort of clustering or compartmentalization. Hence, the deleted region does not have anything to do with the process of partition. This matter was discussed in an informal workshop and it was agreed upon that *a par system secures that each daughter cell is guaranteed at least one plasmid molecule at cell division.* A system that breaks up oligomers (e.g., by site-specific recombination; Nijkamp; Grinter and Barth) or clustering/compartmentalization is not a *par* system.

Low copy-number plasmids (P1, Austin; Abeles et al.; F, Hiraga; R1, Gerdes et al.) possess *par* systems. These seem to code for 2 proteins and a centromer-like cis-acting region. There is randomization during partition; hence, *par* expresses incompatibility (P1, Austin; R1, Gerdes et al.). The synthesis of the ParA protein of plasmid P1 is autoregulated by the ParA protein in cooperation with the ParB protein.

Deletion of various parts of the stability region of plasmid pSC101 gave a series of derivatives (Cohen; Miller et al.). Some of these were stably maintained, but were preferentially lost from cells also containing the wild-type parent plasmid. This phenotype was denoted Cmp^- (competition-negative) and indicates a correlation between replication and stable maintenance. Maybe clustering (dimers, trimers, etc. were not observed in this case) removes the plasmids from the replication pool?

Kunze and Messer observed that *oriC* plasmids are unstably maintained, whereas the plasmid is stably inherited. Hybrids between pSC101 and *oriC* plasmids were found to be unstably maintained.

In order to completely stabilize a low copy-number plasmid, the *par* system is not enough. There is also a system that inhibits cell division in cells carrying only one plasmid copy - the *ccd* (Control of Cell Division) system of plasmid F (Hiraga). Plasmid R1 seems to code for an analogous system (Gerdes et al.). The *ccd* system may be responsible for the induction of the SOS system that is caused by the introduction of UV-damaged λminiF but not by UV-damaged phage λ (Bailone et al.).

In conclusion, stable maintenance of bacterial plasmids is affected by at least 4 different systems:

1. Replication
2. Partition
3. Ccd (Control of Cell Division)
4. Site-Specific Recombination.

In order to be stably maintained, low copy-number plasmids seem to need at least 3 of these. Systems from different plasmids can be combined to give stable inheritance--the cassette model of Hiraga.

ROLE OF THE π INITIATION PROTEIN AND DIRECT NUCLEOTIDE SEQUENCE REPEATS IN THE REGULATION OF PLASMID R6K REPLICATION

Marcin Filutowicz, Michael McEachern, Alan Greener
Pradip Mukhopadhyay, Elliott Uhlenhopp,*
Ross Durland and Donald Helinski

Department of Biology
University of California, San Diego
La Jolla, California 92093

INTRODUCTION

Bacterial plasmids vary considerably in size and copy number. A fundamental property shared by these elements, however, is their stable inheritance in a growing population of bacteria. This stable maintenance at a copy number characteristic of the element is determined by genetic regions on the plasmid that regulate the initiation of replication and subsequent partitioning of the plasmid. As in the case of bacterial and animal viruses that employ different mechanisms for their duplication in a particular host cell, plasmids have evolved different strategies to assure their maintenance at a specific copy number. In some cases both primary and secondary control circuits function to regulate the initiation of plasmid replication events and prevent loss of the element in an exponentially growing bacterial population. This paper will be concerned largely with the regulation of replication of the naturally occurring antibiotic resistance plasmid R6K.

Plasmids With Direct Nucleotide Sequence Repeats at Their Origin

The majority of plasmid elements of Escherichia coli analyzed to date contain direct nucleotide sequence repeats at their origin of replication. This list includes plasmids R6K (1,2), RK2 (3,4), F (5,6), pSC101 (7,8), Rts 1 (9), P1 (10), λdv (11,12) and R485 (D. Stalker, unpub. observ.) (Tab. 1). The direct repeats vary from 17

---

* Current Address: Department of Chemistry, Grinnell College, Grinnell, Iowa.

24 bp in length and are either tandemly joined or separated by e or more nucleotides. In each case, the plasmid also specifies a otein that is required for its replication (Rep protein). The art of the Rep protein structural gene is generally a short dis- nce (25-200 bp) from 1 end of the direct nucleotide sequence re- ats (Tab. 1). In the case of λdv (11), the origin sequence is lo- ted within the O initiation protein gene. The size of the Rep oteins that have been characterized varies from 29,000 to 38,000 In the case of plasmid R6K, the Rep protein (designated π pro- in) has been shown to be required for the initiation of R6K rep- cation (13) and to bind to the direct nucleotide sequence repeats the origin of replication (14,15).

The nucleotide sequences of the direct repeat units for the asmids λdv, R6K, F, RK2, and P1 are displayed in Fig. 1. It is rth noting that with few exceptions, the hexanucleotide TGAGPuG is esent in each of the direct repeats. While the role of this hexa- cleotide sequence in plasmid DNA replication remains to be deter- ned, it appears that for plasmid R6K, the G occupying the second d fourth positions is essential for binding of the π initiation otein and a functional replication origin (see section below). e important role of the direct repeats in the regulation of asmid DNA replication is also indicated by the finding that the epeat sequences of plasmids F (16,17), P1(10), R6K (S. Yang, unpub. serv.) and RK2 (T. Schmidhauser, M. deSmet, and P. Reeves, unpub. serv.), when inserted into another plasmid replicon, express in- mpatibility properties characteristic of the parent plasmid.

eneral Features of R6K Replicon

The origins of replication, designated α, β, and γ, are con- ined within a 4-kb region of the 38-kb plasmid R6K (Fig. 2) (18-). The α and β origins display in E. coli sequential, bi-direc- ional replication toward an asymmetrically located terminus 8,19). An in vitro system, capable of replication of R6K or its

b. 1. Direct nucleotide sequence repeats at their origin of rep - lication.

Plasmid	Direct Repeats at Origin (A)	Replication Protein (mol. wt.) (B)	Distance Between Genetic Regions A and B	Ref.
R6K	7-22 bp	35,000	207 bp	1, 2
pSC101	3-18 bp	37,500	95 bp	7, 8
Rts1	5-24 bp	33,000	26 bp	9
F	4-19 bp	29,000	98 bp	5, 6
P1	5-19 bp	32,000	56 bp	10
RK2	8-17 bp	32,000	~4000 bp	3, 4

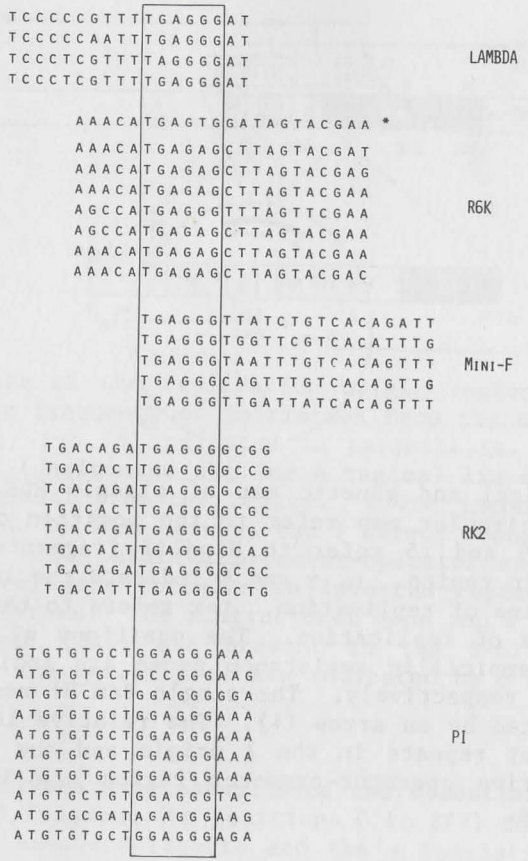


Fig. 1 Families of direct repeat units from the origin region of 5 different plasmid replicon sequences are aligned to indicate the location of a 6-bp homologous region. The 8th direct repeat of plasmid R6K that is in the putative promoter-operator region of the π structural gene is indicated (*). The lambda direct repeats are present in the origin region of the λdv plasmid. Reference citations for these sequences are given in the text.

derivatives, has been developed from E. coli cells (13,22). A minimal replication region, consisting of 2 separable components: the γ origin and the structural gene for the π initiation protein (pir), has been defined (23). The γ origin contains 7 tandemly associated 22-bp direct repeats (1). The eighth copy of this repeat is located in the putative promoter-operator region of the pir gene (2,24) and is likely to play a role in the autoregulation of expression of the pir gene (2,25).

The π initiation protein is also required for the initiation of replication from the α and β origins (26). However, unlike the γ

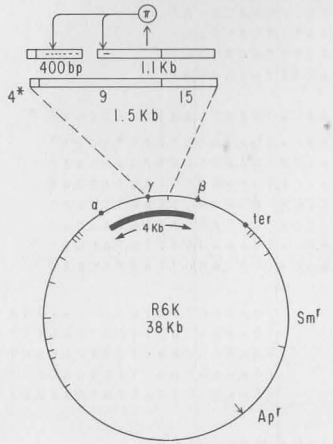


Fig. 2. Physical and genetic map of plasmid R6K. Hash marks on the circular map refer to the position of HindIII sites. 4*, 9 and 15 refer to HindIII fragments present in the origin region. α, γ and β indicate the locations of the 3 origins of replication. ter refers to the assymetric terminus of replication. The positions of the streptomycin and ampicillin resistance genes are indicated by Sm^r and Ap^r, respectively. The single Bam Hl restriction site is located by an arrow (↓). The relative locations of the 7 direct repeats in the λ origin and the 8 repeats in the putative operator-promoter region are indicated by dash marks.

origin it has not been possible to isolate the α or β origin as a short segment of DNA capable of replication when the π protein is provided in trans. As indicated in Fig. 3, the minimal size of the functional α and β origins obtained to date is approximately 2 kb (24,26). The complete nucleotide sequence of the region containing the γ and β origins of replication has been determined (1,2,24,26). This region also includes a functional promoter [(27) and P. Mukhopadhyay, unpub. observ.] specifying transcription in the direction of the repeats, the seven 22-bp direct nucleotide sequence repeats that express R6K incompatibility (designated incA, S. Yang, unpub. observ.), a second incompatibility region (designated IncB; S.Yang, unpub. observ.) the pir gene and a second open reading frame (ORF) (26).

The γ origin has been delineated by Tn5 transpositional analysis (28). It consists of a 260-bp region extending from a short distance to the left of the junction of HindIII fragments 4 and 9 (Fig. 2 and 3) to just before the blgII site in HindIII fragment 9. Removal of 3 or more of the direct repeats from the γ origin results in loss of the origin activity (28). As indicated in Fig. 4, several deletions and insertions have been produced in the β-origin

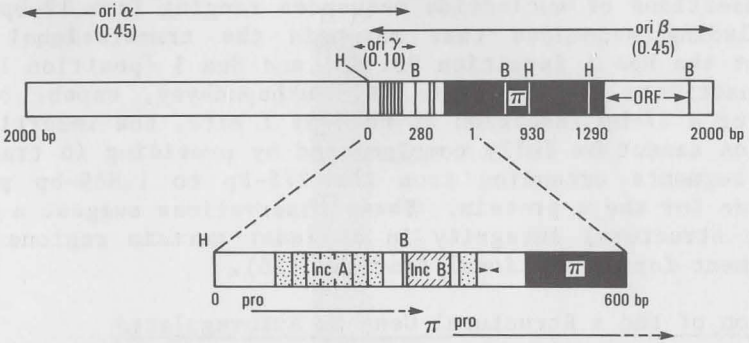


Fig. 3. Structure of the replication origin region of R6K. The relative frequency of initiation from the α, γ and β origins in vivo is indicated in parenthesis. The 7 direct repeats (designated the Inc A region) lie downstream from a functional promoter region. A 2nd incompatibility region (Inc B) lies between the 7 direct repeats and the 8th repeat. The putative promoter-operator region of the π structural gene contains an inverted repeat indicated by facing arrows. The π structural gene and a 2nd open reading frame (ORF) are present in the β-origin fragment. HindIII and BglII sites are indicated by H and B, respectively.

region. These analyses have established the essential role of both the HindIII-Bgl II fragment (bp positions 0 to 277) containing the 7 direct nucleotide sequence repeats and the π initiation protein in the activity of both the α and β-origins of replication. In addi-

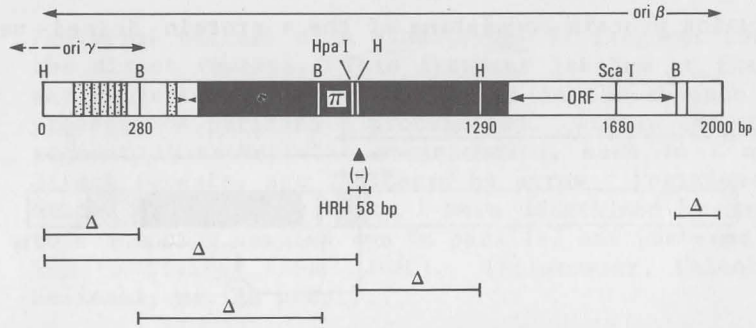


Fig. 4. The position of deletions and insertions in the β-origin fragment (26). The extent of each deletion is indicated by cross bars. HRH refers to a HindIII fragment of 58 bp that contains an internal Eco RI site. Other designations are described in the legend to Fig. 3.

tion, insertions of nucleotide sequences ranging from 12-bp to 340-bp (including sequences that maintain the translational reading frame) at the Hpa I (position 931-bp) and Sca I (position 1,680-bp) sites inactivate the β origin (P. Mukhopadhyay, unpub. observ.). Except for a 12-bp insertion at the Hpa I site, the insertional inactivation cannot be fully complemented by providing in trans the 2 Bgl II segments extending from the 275-bp to 1,869-bp positions which code for the π protein. These observations suggest a requirement for structural integrity in at least certain regions of this 2-kb segment for a functional β origin (26).

Expression of the π Structural Gene is Autoregulated

Plasmids carrying a fusion of the pir-promoter to the lacZ gene express β-galactosidase activity in E. coli at a substantially reduced level when the π protein is provided in trans [(25) and G. Davis and M. Filutowicz, unpub. observ.]. The activity of the π protein in repressing the expression of the pir gene has also been demonstrated in vitro utilizing purified π protein and E coli RNA polymerase (A. Greener, unpub. observ.). This activity of the π protein, illustrated in Fig. 5, has been examined also by the DNase I footprint technique (15) and M. Filutowicz, unpub. observ.]. It was found that purified π protein and RNA polymerase bind to the presumed operator-promoter region of the π structural gene. As shown in Fig. 6, the binding sites overlap for 3 helical turns that include 2 partial repeat sequences in an inverted orientation and adjacent to the eighth repeat. Competition binding studies further demonstrated that the π protein will displace prebound RNA polymerase. These results strongly support a mechanism for autoregulation of the pir gene that involves competition between the π protein and RNA polymerase for binding to the pir promoter region.

Mutations in the Direct Nucleotide Sequence Repeats Alter π Protein Binding and Inactivate the γ-origin

A fusion protein consisting of the π protein joined near its

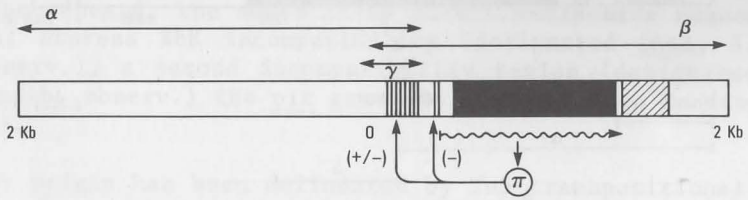


Fig. 5. Interaction of the π protein with the direct nucleotide sequence repeats. The relative positions of the pir gene (solid region) and the ORF (diagonal lines) are indicated with respect to the α, γ and β origins of replication. The narrow vertical boxes locate the positions of the direct repeats.



Fig. 6. Footprint pattern of a HindIII-Bgl II fragment containing the direct repeats. This fragment labeled at the 3' end was subjected to DNase I cleavage in the absence (a) and presence of purified π protein (b). Within the protected segment characteristic enhancements, each in 1 of the 7 direct repeats, are indicated by arrow. Positions of enhanced cleavages by DNase I were determined by comparison to sequencing samples run in parallel and numbered according to Stalker et al., (2). (Filutowicz, Uhlenhopp and Helinski, ms. in prep.).

C-terminal end to an N-terminal α-donor polypeptide of E. coli β-galactosidase has been shown to bind to the 7 tandem repeats within the γ-origin (14,15). DNase I footprint analysis,of this specific binding using purified wild-type π protein exhibits a similar

association with the tandem repeats (Fig. 7) as shown by the chimeric protein (M. Filutowicz, unpub. observ.). Sodium bisulfite in vitro mutagenesis of the direct repeat region within the γ-origin was carried out to assess the primary structural requirements for π binding and origin activity. The mutagenized γ-origin, joined to a pBR322 replicon, was analyzed for functionality of the γorigin in a polA214 temperature-sensitive host strain. A number of partial or completely defective γ-origin mutants have been obtained (M. McEachern, unpub. observ.). The mutational changes, identified by nucleotide sequence analysis are shown for mutants γ117 and γ120 in Fig. 8. Mutant γ117, containing G to A transitions at 3 positions of the first nucleotide sequence repeat, exhibits defective γ-origin activity and loss of binding of the wild-type π protein specifically to this altered repeat (M. McEachern, unpub. observ.). Two of the 3 G to A substitutions are in the consensus hexanucleotide sequence shown in Fig. 1. A revertant of this γ-origin mutant also has been obtained (M. McEachern, unpub. observ.). This revertant of γ117 was deleted for the altered first repeat. It is of interest that this first repeat is immediately downstream from the active promoter in the γ-origin region (Fig. 3 and 8). Since E. coli rpoB mutants have been found to suppress temperature-sensitive π protein mutants (M. Filutowicz, unpub. observ.), defective binding of the π protein to the first repeat in γ117 may prevent a specific interaction of the π protein with RNA polymerase at the γ-origin promoter site which may be required for origin activity. Deletion of this mutant repeat may restore the structural requirements for this specific interaction of the π protein and RNA polymerase.

Mutant γ120 contains 5 C to T transitions. DNase I footprint analysis showed that π was unable to bind to the altered sixth repeat and bound less efficiently to the altered seventh repeat (M. McEachern, unpub. observ.). A revertant of γ120 contained a deletion of the sixth repeat, suggesting again that spacing requirements

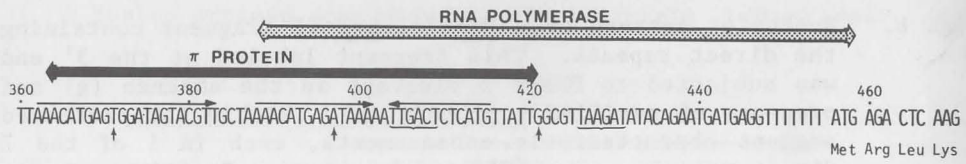


Fig. 7. π protein and RNA polymerase binding sites in the promoter-operator region of the pir gene. The coordinates are taken from Stalker et al., (2). The putative -10 and -35 segments of the pir are underlined. The horizontal arrows indicate the 8th direct and the inverted repeats which show partial homology with the repeat of 22 bp. The vertical arrows indicate position of enhanced DNase I cleavage frequencies observed in the presence of the π protein. (Filutowicz, Davis, Greener and Helinski, ms. in prep.).

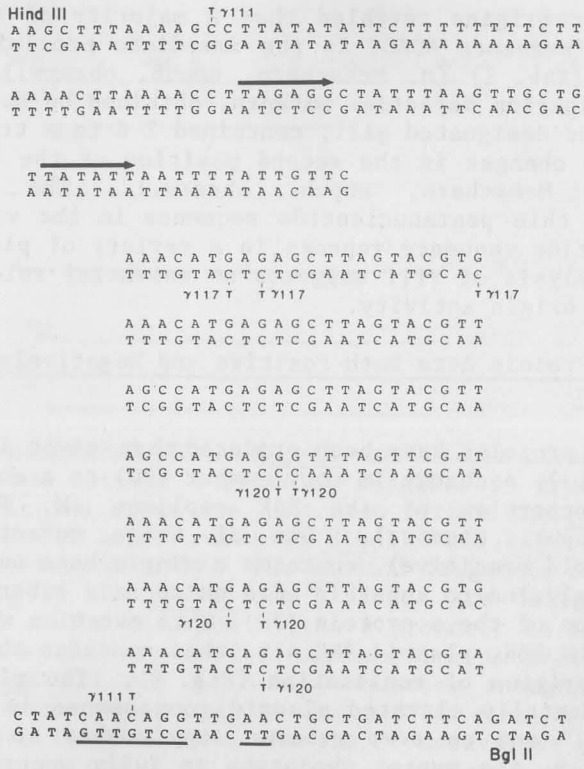


Fig. 8. Location of bisulfite-induced mutations resulting in an inactivation of the γ-origin. The sequence of the HindIII-Bgl II segment is taken from Stalker et al., (1). The horizontal arrows indicate putative -10 and -35 sequences of a promoter located upstream of the seven 22-bp repeats [(27) and P. Mukhopadhyay, unpub. observ.]. Solid bars indicate conserved sequences present in several plasmids containing direct repeats. (McEachern, Filutowicz and Helinski, ms. in prep.).

are critical to γ origin functionality. Thus, in the case of γ-origin mutants γ117 and γ120 base changes at the seventh and ninth positions within the 22-bp repeat (and within the consensus hexanucleotide sequence) result in a loss of π binding to the altered repeat. It is worth noting that the bases at these positions are among those that interact with the π-β-galactosidase fusion protein (15).

A γ-origin Mutant Contains a Base Substitution in a Consensus ACAGG Sequence

Analysis of plasmids containing direct nucleotide sequence re-

peats at their origins revealed that a majority of these plasmids contained the sequence ACAGG in the immediate proximity of the direct repeats (Tab. 2) (M. McEachern, unpub. observ.). One of the γ-origin replication defective mutants, obtained by sodium bisulfite mutagenesis and designated γ111, contained 2 G to A transitions with 1 of the base changes in the second position of the ACAGG sequence (Fig. 8) (M. McEachern, unpub. observ.). The frequency of occurrence of this pentanucleotide sequence in the vicinity of the direct nucleotide sequence repeats in a variety of plasmids and the mutational analysis of γ111 suggests an essential role for this sequence in R6K origin activity.

π Initiation Protein Acts Both Positive and Negatively at the Origin of Replication

Mutant π proteins have been produced that result in temperature-sensitivity (13), a change in copy number (30) or a change in incompatibility properties of the R6K replicon (M. Filutowicz, M. McEachern, unpub. observ.). One of these mutants, designated pir405-Cos (cold sensitive), contains a single base substitution resulting in a glycine to aspartic acid amino acid substitution at the 8first position of the π protein (30). This mutation was produced in a derivative of R6K, plasmid PRK 419, that contains the pir gene and the β and λ-origins of replication (Fig. 9). The pir405-Cos exhibits a substantially elevated plasmid copy number in E. coli cells growing at 42° and an even greater copy number at 30° (30). As shown in Fig. 9, the mutant phenotype is fully suppressed when the wild-type pir sequence is provided in trans. Complete suppression requires the N-terminal and at least part of the C-terminal sequence of the π protein (30). A second π structural gene mutant,

Tab. 2. Conserved pentanucleotide sequence in the origin region of plasmids containing direct repeats. The sequences were taken from Ref. 1 (R6K), 29 (pSa), 3 (RK2), 9 (Rts1), and 5 (F). The sequence for plasmid R485 was obtained by D. Stalker (unpub. observ.).

R6K	t a c t a t C a A C A G G t t g A A c t g c t
R485	g t a c a t C C A C A G G c t a A A a a a a t
pSa	a c c c g g a g A C A G G c a t A A t a a t a
RK2	g g c t g t C C A C A G G c a g A A a a t c c
Rts1	g c c a a a C C A C A G G c a c A c t t a t t
F	a a t t t g t C A C A G G g t t A A g g g c a

Conserved pentanucleotide sequence in the origin region of plasmids containing direct repeats. The sequences were taken from references 1 (R6K), 29 (pSa), 3 (RK2), 9 (Rts1), and 5 (F). The sequence for plasmid R485 was obtained by D. Stalker (unpublished observations).

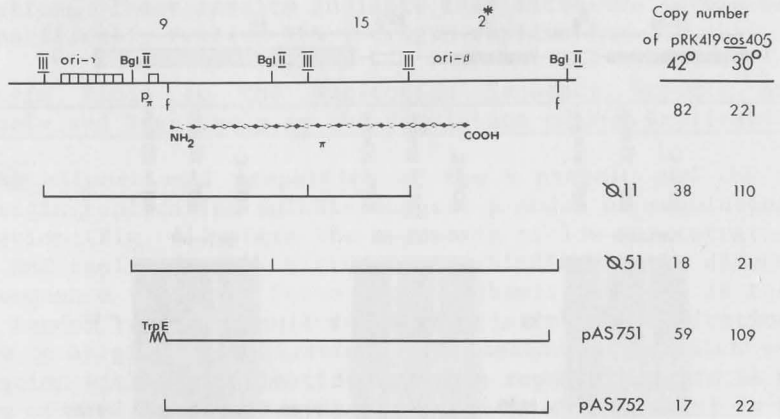


Fig. 9. Copy number of pRK419 pir405-cos in the presence of wild-type and altered π proteins supplied in trans. Relevant restriction sites are indicated within the pir structural gene. III and f refer to HindIII and Hinf I restriction sites, respectively. The HindIII fragments of R6K present in pRK419 and pir405-cos plasmids are designated 9, 15, and 2*. The location of the π promoter is designated p_π, whereas the eight 22bp repeats are indicated by boxes. Ø11 and Ø51 are π coding sequences integrated into the host chromosome. pAS751 and pAS752 contain π coding sequences inserted into the pBR322 derivative pAS620. TrpE refers to the 7 NH_2-terminal amino acid residues of the trpE gene and the tryptophan operon promoter. The copy number of pRK419 pir405-cos was measured in 10-ml cultures grown at 42°C and in cultures that have been shifted from 42°C to 30°C for 4 hr. Linearized pRK248 was the internal standard in strains Ø11 and Ø51. Data were presented in Ref. 30.

designated pir13-Cop and resulting in a leucine to arginine substitution at the 9 first amino acid position of the π protein, similarly exhibits a high copy-number phenotype that is suppressed by a wild-type pir sequence provided in trans (M. McEachern, unpub. observ.). The pir13-Cop or pir405-Cos mutation, present in plasmid pRK 419, also results in a decrease in R6K incompatibility (M. McEachern and M. Filutowicz, unpub. observ.).

The properties of the pir405-Cos and pir13-Cop mutants indicate that the π protein plays a role in the negative control of R6K replication in addition to its positive requirement for the initiation of plasmid replication. This negative activity of π is also supported by pulse-labeling experiments that measure R6K γ-origin activity in E. coli cells after induction of π synthesis. As shown in Fig. 10, a pBR322 hybrid plasmid containing a λ cI-ts gene and the R6K pir gene downstream from the λ P_R promoter was constructed and

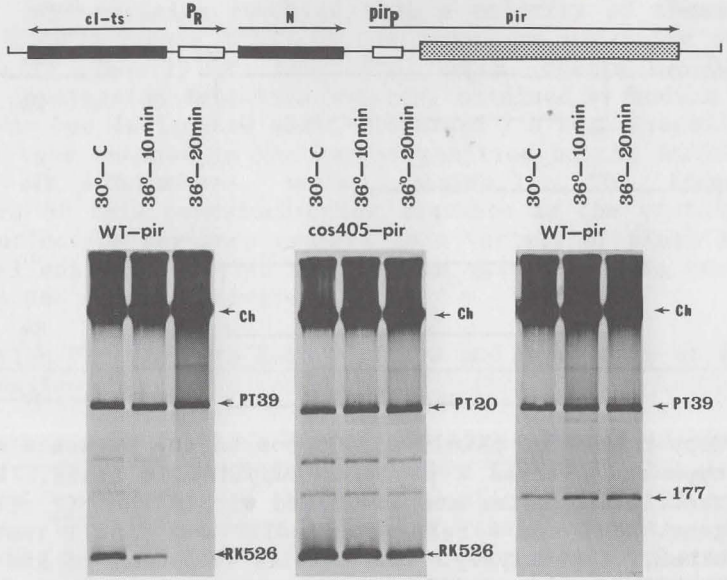


Fig. 10. The effect of increased intracellular π concentration on the replication of a γ origin plasmid. Plasmids pPT39 and pPT20 contain a FnuDII-pir fragment from wild-type pRK419 and the pir405-cos mutant, respectively, placed downstream of an inducible P_R-promoter of phage λ. The key element involved in control of the π protein expression in these constructs are schematically shown at the top of the figure. E. coli strain P678-54 (Thy-) was transformed at 30°C with appropriate combinations of plasmids. 2 ml of logarithmically growing cultures (2×10^8 cells ml^{-1}) supplemented with 5 μg ml^{-1} of thymidine were labeled with [2^{14}C]-thymidine (1 μci ml^{-1}) for 15 min. The cultures were subjected to a temperature shift (35°) and then transferred back to 30°C after the time indicated and before the addition of radioisotope. The cells were harvested and washed with ^{14}C-free medium. The plasmid DNA molecules were separated by agarose gel electrophoresis. RK526 and 177 refer to the R6K γ-origin plasmid and pACY§c177, respectively.

used to temperature-induce wild-type π protein synthesis in E. coli (P. Tooker and M. Filutowicz, unpub. observ.). Replication of the λ-origin plasmid pRK 526 in cells carrying the inducible pir plasmid was measured by pulse labeling DNA with C^{14} thymidine before and after temperature induction of π synthesis results in a substantial reduction of γ-origin replication (Fig. 10). As a control, the replication of plasmids pACYC177, R300B, PB§t322 and an RK2 derivative were not affected by the induction of π synthesis. Finally, using analogous constructs, the induction of mutant pir405-Cos π synthesis appeared to be considerably less effective in repressing γ-origin

replication. These results indicate that excessive levels of π protein specifically repress R6K γ-origin replication.

π Protein Binds to the Nucleotide Sequence Repeats and Acts Positively and Negatively in the Regulation of R6K Replication

The bifunctional properties of the π protein and the analysis of γ-origin replication mutants suggest a model of regulation of R6K replication (Fig. 11) where the π protein at low concentrations initiates R6K replication by virtue of its binding to the direct nucleotide sequence repeats. Subsequent biochemical events in the region of the tandem repeats result in the initiation of replication at the α, β or γ origin of replication. The mechanism by which π protein association with the nucleotide sequence repeats results in the initiation of replication at a distant site (α or β origin) is unknown. Some structural integrity involving the nucleotide sequence between the direct repeats and the β-origin appears to be required for a functional β-origin. According to this model at higher π concentrations a negative activity of π is exerted at both regions containing repeats of 22 bp; the γ-origin and the operator region for the pir gene. The range of π concentrations required for positive or negative activity is unknown. In an earlier study involving a functional π protein lacking several N-terminal amino acids, the results indicated a constant copy number of a γ-origin replicon over a broad range of π concentrations (25). The role of autoregulation of the pir gene expression and the role of the promoter upstream from the direct nucleotide sequence repeats in the regulation of R6K replication are also unknown.

CONCLUDING REMARKS

The display of different strategies for the regulation of replication of plasmid elements underscores the versatility with which

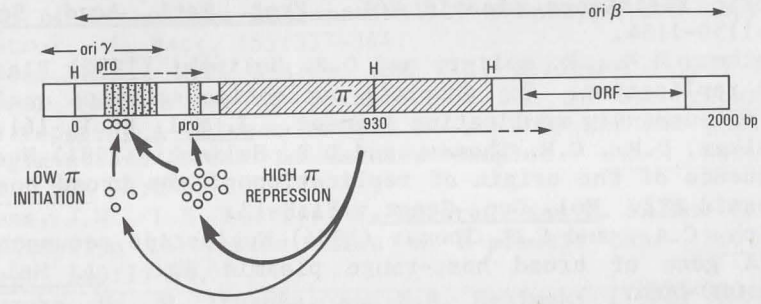


Fig. 11. Model of regulation of R6K replication. The circles represent π protein molecules. The various designations are described in the legend to Fig. 3.

plasmid elements have employed basic components (origin sequences, replication proteins, trans-acting repressors) in evolving regulatory circuits that provide for a fixed copy number in a bacterial host. In several of the plasmid systems examined, this copy number is the result of the interplay of separable positive- and negative-acting elements. In the case of plasmid R6K, both positive and negative activities reside in a single component, the π protein. This protein recognizes and binds to a multiple set of 22-bp nucleotide repeats. Since mutations that eliminate binding of the protein to a single repeat abolish replication activity at the origin sequence, this interaction appears to be critical for initiating a round of plasmid replication. The association of the π protein with the direct repeats most often results in replication origin activity at a considerable distance from the repeats. The structural gene for the π protein is autoregulated by a mechanism involving competition between the π protein and RNA polymerase for the putative promoter operator region of the pir gene. This region contains nucleotide sequences similar to the direct repeats at the origin of replication. The majority of plasmids in E. coli share with R6K the property of nucleotide sequence repeats at the origin of replication and a plasmid-specified replication protein. It will be of interest to determine if similarities exist among these plasmids in the nature of the interaction of the replication protein with these nucleotide sequence repeats.

ACKNOWLEDGMENT

This work was supported by grants from the National Institutes of Allergy and Infectious Diseases and the National Science Foundation. E.U. was supported by the National Science Foundation College Faculty Program.

REFERENCES

1. Stalker, D.M., R. Kolter, and D.R. Helinski (1979) Nucleotide sequence of the region of an origin of replication of the antibiotic resistance plasmid R6K. Proc. Natl. Acad. Sci., USA 76:1150-1154.
2. Stalker, D.M., R. Kolter, and D.R. Helinski (1982) Plasmid R6K DNA replication. I. Complete nucleotide sequence analysis of an autonomously replicating segment. J. Mol. Biol. 161:33-43.
3. Stalker, D.M., C.M. Thomas, and D.R. Helinski (1981) Nucleotide sequence of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:8-12.
4. Smith, C.A., and C.M. Thomas (1984) Nucleotide sequence of the trfA gene of broad host-range plasmid RK2. J. Mol. Biol. 175:000-000.
5. Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami (1981) Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene 15:257-271.
6. Tolun, A., and D.R. Helinski (1982) Separation of the minimal replication region of the F plasmid into a replication origin segment and a trans-acting segment. Mol. Gen. Genet. 186:372-377.
7. Churchward, G., P. Linder, and L. Caro (1983) The nucleotide sequence of replication and maintenance functions encoded by plasmid pSC101. Nucl. Acids Res. 11:5645-5659.
8. Armstrong, K.A., R. Acosta, E. Ledner, Y. Machida, M. Pancotto, M. McCormick, H. Ohtsubo, and E. Ohtsubo (1984) A 37 kilodalton plasmid-encoded protein is required for replication and copy number control in pSC101 and Ts derivative pHS1. J. Mol. Biol. (in press).
9. Kamio, Y., A. Tabuchi, Y. Itoh, H. Katagiri, and Y. Terawaki (1984) Complete nucleotide sequence of mini-Rts1 and its copy mutant. J. Bact. 158:307-312.
10. Abeles, A., K.M. Snyder, and D. Chattoraj (1984) P1 plasmid replication: replicon structure. J. Mol. Biol. 173:307-324.
11. Scherer, G. (1978) Nucleotide sequence of the O gene and of the origin of replication in bacteriophage λ DNA. Nucl. Acid Res. 5:3141-3156.
12. Grosschedl, R., and G. Hobom (1979) DNA sequences and structural homologies of the replication origins of lambdoid bacteriophages. Nature. 277:621-627.
13. Inuzuka, M., and D.R. Helinski (1978) Replication of antibiotic resistance plasmid R6K DNA in vitro. Biochem. 17:2567-2573.
14. Germino, J., and D. Bastia (1983) The replication initiator protein of plasmid R6K tagged with β-galactosidase shows sequence-specific DNA binding. Cell 32:131-140.
15. Germino, J., and D. Bastia (1983) Interaction of the plasmid R6K-encoded replication initiator protein with its binding sites on DNA. Cell 34:125-134.
16. Tolun, A., and D.R. Helinski (1981) Direct repeats of the F plasmid IncC region expresses F incompatibility. Cell 24:687-694.
17. Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsubara (1983) Role of 9 repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bact. 155:337-344.
18. Lovett, M.A., R.B. Sparks, and D.R. Helinski (1975) Bidirectional replication of plasmid R6K DNA in Escherichia coli: correspondence between origin of replication and position of single-strand break in relaxed complex. Proc. Natl. Acad. Sci., USA 72:2905-2909.
19. Crosa, J.H., L.K. Luttrop, F. Heffron, and S. Falkow (1975) Two replication initiation sites on R-plasmid DNA. Molec. Gen. Genet. 140:39-50.
20. Inuzuka, N., M. Inuzuka, and D.R. Helinski (1980) Activity in vitro of 3 replication origins of the antibiotic resestare plasmid RSF 1040. J. Biol. Chem. 255:11071-11074.

21. Crosa, J.H. (1980) Three origins of replication are active in vivo in the R-plasmid RSF 1040. J. Biol. Chem. 255:11075-11077.
22. Inuzuka, M., and D.R. Helinski (1978) Replication of antibiotic resistance plasmid R6K DNA in vitro. Biochemistry 17:2567-2573.
23. Kolter, R., M. Inuzuka, and D.R. Helinski (1978) Trans-complementation dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell 15:1199-1208.
24. Shon, M., J. Germino, and D. Bastia (1982) The nucleotide sequence of the replication origin of the plasmid R6K. J. Biol. Chem. 257:13823-13827.
25. Shafferman, A., R. Kolter, D.M. Stalker and D.R. Helinski (1982) Plasmid R6K DNA replication. III. Regulatory properties of the π initiation protein. J. Mol. Biol. 161:57-76.
26. Shafferman, A., and D.R. Helinski (1983) Structural properties of the beta origin of replication of plasmid R6K. J. Biol. Chem. 258:4083-4090.
27. Shafferman, A.L., and D.R. Helinski (1984) Transcription signals in a region essential for replication of plasmid R6K. Plasmid (submitted for publication).
28. Kolter, R., and D.R. Helinski (1982) Plasmid R6K DNA replication. II. Direct nucleotide sequence repeats are required for an active gamma origin. J. Mol. Biol. 161:45-56.
29. Tait, R.C., C.I. Kado, and R.L. Rodriguez (1983) A comparison of the origin of replication of pSa with R6K. Mol. Gen. Genet. 192:32-38.
30. Stalker, D., M. Filutowicz, and D.R. Helinski (1983) Release of initiation control by a mutation in the plasmid encoded protein required for R6K DNA replication. Proc. Natl. Acad. Sci., USA 80:5500-5505.

INITIATION OF REPLICATION OF THE ESCHERICHIA COLI CHROMOSOMAL ORIGIN RECONSTITUTED WITH PURIFIED ENZYMES

Jon M. Kaguni,[1] LeRoy L. Bertsch, David Bramhill, James E. Flynn,[2] Robert S. Fuller,[3] Barbara Funnell, Satoko Maki, Tohru Ogawa, Kazuko Ogawa, Arie van der Ende, and Arthur Kornberg

Biochemistry Department of
Stanford University
Stanford, California 94306

ABSTRACT

A mixture of purified proteins has replaced a crude enzyme fraction capable of efficient replication of oriC-containing plasmids. The reconstituted enzyme system contains proteins which provide initiation, replication, and specificity functions required for dnaA-dependent replication specific for an oriC template. Replication can be separated into successive stages of RNA synthesis and DNA replication. Isolation of an intermediate no longer requiring RNA polymerase action requires the presence of dnaA protein, DNA gyrase, dnaB protein and dnaC protein. Intermediate formation likely involves binding of dnaA protein to a 9-bp sequence present 4 times as inverted repeats within the chromosomal origin sequence.

INTRODUCTION

The study of replication of single-stranded DNA bacteriophages M13, G4, and ϕX174 has contributed significantly to our current

[1]Present address: Biochemistry Dept., Michigan State University, East Lansing, Michigan 48824; [2]deceased; [3]present address: Dept. of Microbiology and Immunology, University of California, Berkeley, California 94720.

understanding of priming of nascent chains in E. coli (1). These bacteriophages rely almost entirely on host-encoded enzymes; their modes of replication represent 3 distinct mechanisms of priming DNA chains, all extended by DNA polymerase III holoenzyme, the major replicative enzyme of E. coli (2). Such studies, although useful in understanding nascent chain synthesis, do not explain how replication is initiated at the unique chromosomal origin, oriC, nor how this process is regulated and coupled to other cellular events.

Understanding the biochemical nature of the initiation process and its regulation has become accessible because of 2 advances. The first is the availability of recombinant DNAs containing the E. coli chromosomal origin, oriC (3,4). This unique sequence of 245 bp, highly conserved among many gram-negative bacteria (5), has been well characterized by deletion, mutation, and comparative DNA sequence analyses (6). The second advance is the discovery of a soluble enzyme system which efficiently replicates such recombinant DNAs in a bidirectional manner by initiating DNA synthesis from or near oriC (7,8).

Further studies, focused on resolving this enzyme system into its component parts, have identified 3 functional classes of proteins: (i) initiation proteins involved in recognizing the oriC sequence and transcribing it, (ii) replication proteins involved in extension of DNA chains, and (iii) specificity proteins which suppress DNA synthesis at extraneous sites throughout the DNA duplex while permitting it to occur at the oriC sequence. A key feature of the initiation process likely involves recognition of specific repeated sequences within oriC by dnaA protein.

A reconstituted mixture of purified proteins has been developed which performs initiation, replication and specificity functions (9). Replication of a variety of oriC-containing DNAs occurs efficiently and can be separated into distinct stages of RNA synthesis and DNA replication. A biochemical understanding of the initiation process and hopefully its regulation can now be approached.

dnaA PROTEIN BINDING SITES

The product of dnaA has been implicated in genetic and physiological studies at an early stage in initiating a cycle of chromosomal replication (10). With the exception of pSC101 (11,12), this dnaA requirement is unique for replication from the E. coli origin, oriC. The dnaA protein has been purified based on its ability to bind to restriction fragments containing oriC (13) and by a complementation assay for in vitro replication of oriC-containing templates in conjunction with its overproduction in an expression vector (14).

As judged by nitrocellulose filter retention assays, dnaA protein specifically binds to restriction fragments containing the oriC sequence. DNase I protection experiments indicate that dnaA protein specifically binds to and protects a 9-bp sequence found 4 times in oriC (15). Protection by dnaA protein appears to be cooperative. At levels of dnaA protein of less than 20 molecules per DNA fragment, little or no protection was observed; a level of 30 to 40 is effective in protecting the repeat sequences as well as adjacent sequences. Sites of enhanced cleavage are observed within some repeat sequences as well as outside these repeats (15).

This sequence of 9 bp is not unique to the chromosomal origin; homologous sequences are found in a variety of DNAs (Tab. 1). In addition to binding to oriC, dnaA protein also binds to restriction fragments containing: (i) the origin regions of pBR322, ColE1, and

Tab. 1. Consensus Sequence of dnaA Protein Binding Sites (15).

A. Sequences in known binding sites:	
oriC R1	TTG TTATCCACA GGC
oriC R2	GGG TTATACACA ACT
oriC R3	TAG TTATCCAAA GAA
oriC R4	GAG TTATCCACA GTA
ori-pBR322	CGG TTATCCACA GAA
ori-ColE1	CAG TTATCCACA GAA
ori-pSC101	GAG TTATACACA GGG
dnaA promoter region	GAT TTATCCACA GGA
dnaA structural gene	AGT TTTTCCACA CCT
"X-protein" promoter region	TAC TTTTCCACA GGT
IR_L-Tn5	CTC TTATACACA AGT
M13 site	GCG TTATACAAA TCT
Consensus:	gag TTAT$^{C}_{A}$CA$^{C}_{A}$A gga
B. Other homologous sequences:	
Origins of replication:	
R100	TGC TTATCCACA TTA
R1	TGC TTATCCACA TTT
CloDF13	AAG TTACCCACA GAT
P1	CAC TTATCCACA TTA
P1	ACA TTATCCACT GGA
F	GGG TTATCCACT TAT
F	CAC TTATCCACG GGG
Regulatory regions:	
pyrBI (aspartate transcarbamylase)	ACC TTATCCAAA CTG
argF (ornithine transcarbamylase)	GAT TTATACAAA AAA
polA	AAA TTATCCACA TGA
uvrB	TGG TTATCCACA GGA
dam	GCT TTCTCCACA GCC

pSC101; (ii) the promoter regions for the dnaA protein and the "X-protein"; (iii) the dnaA structural gene; (iv) the left inverted repeat of Tn5; and (v) a coding region of M13. Other homologous sequences presumably bound by dnaA protein are found in origin regions of phage P1 and plasmids R100, R1, ColDF13, and F, as well as in regulatory regions for pyrB1, argF, polA, uvrB and dam.

These numerous dnaA binding sites may be important in regulating replication or gene expression. Support for such roles comes from the following lines of evidence: dnaA protein is required for replication of the E. coli chromosome (7,10), replication of pSC101 (11,12) and transposition of Tn5 (Johnson and Reznikoff, pers. comm.); dnaA protein may autoregulate its own expression (16).

INITIATION OF REPLICATION

Reconstitution of oriC Replication

A reconstituted enzyme system has been developed which depends on dnaA protein and efficiently replicates a variety of oriC-containing DNAs which differ both in the orientation of the oriC-containing fragments relative to the vector and in the amount of chromosomal DNA flanking oriC contained in the DNA fragment.

Maximal DNA synthesis is observed with these recombinant DNAs upon addition of partially purified fractions (resolved by chromatography of an ammonium sulfate fraction on an Amicon dye affinity column) to a mixture of highly purified proteins. In the presence of topoisomerase I (17), RNase H (18), and HU protein (19), which ensure that DNA synthesis is specific for oriC-containing DNA templates, replication is efficient and depends on dnaA protein. The activities contained in these partially purified fractions are currently being further purified in order to understand how they stimulate replication.

Replication of M13oriC2LB5 RF Requires Many Purified Proteins

A recombinant DNA, M13oriC2LB5 (Fig. 1), containing the minimal oriC sequence of 245 bp within a 327-bp fragment replicates efficiently in the presence of purified enzymes (Tab. 2). Requirements are observed for RNA polymerase, the gyrase B subunit, dnaA protein, single-strand DNA binding protein (SSB), dnaB protein, dnaC protein and DNA polymerase III holoenzyme. The incomplete dependence on protein HU for DNA synthesis may be due to its presence in other enzyme fractions. Poor dependencies on primase and gyrase A subunit are also observed. Based on specific assays for primase, it seems unlikely that this activity is present in significant amounts in other enzyme fractions. One possibility is that RNA polymerase may replace the requirement for primase in priming DNA chains for lagging strand synthesis. Alternatively, this reaction may represent

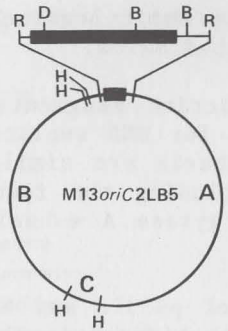


Fig. 1. Restriction map of M13oriC2LB5. This M13 chimera contains a 327-bp HaeIII-AccI fragment containing oriC with EcoR1 linker ends from pTOA7 (T. Ogawa and A. Kornberg, unpub. data) inserted into the vector M13ΔE101. ■, oriC; H, HaeII; R, EcoR1; D, HindIII; B, BamH1.

an uncoupling of leading and lagging strand synthesis as has been observed for ColE1 replication (20).

Despite the poor dependence on the gyrase A subunit, its participation is inferred by inhibition by oxolinate, the specific

Tab. 2. Requirements for replication of M13oriC2LB5 RF in the presence of PVA.*

Component omitted	DNA synthesis (pmol)
Expt. 1:	
None	541
RNA polymerase	26
Gyrase A subunit	512
Gyrase B subunit	22
dnaA protein	64
Expt. 2:	
None	762
Protein HU	456
SSB	105
dnaB protein	23
dnaC protein	54
Primase	374
Pol III holoenzyme	5

*Topoisomerase I and RNase H were present to maintain oriC template specificity and dnaA dependence.

gyrase A inhibitor, and by the dependence on gyrase A under other reaction conditions described below.

Interestingly, restriction fragments containing the oriC sequence serve as templates for DNA replication (Tab. 3). Enzymatic requirements for DNA synthesis are similar to those of a reaction with supercoiled DNA, including the requirement for the gyrase B subunit (Tab. 4) and the gyrase A subunit (inferred by oxolinate-sensitivity).

Whereas replication of pSY317 and M13oriC26 RF started at or near the oriC sequence with bidirectional progress with a crude enzyme system (8), replication of M13oriC2LB5 RF is principally unidirectional from oriC in the purified enzyme system. It is not yet clear whether the lack of bidirectional progress is due to a lack of enzymes or certain DNA sequences, or both. The fewer priming events implied for a unidirectional mode of replication may simplify analysis of the initiation event.

The absolute need in oriC replication for a high concentration of a hydrophilic polymer such as polyvinyl alcohol or polyethylene

Tab. 3. Replication of restriction fragments containing oriC.

	Template (pmol)	DNA synthesis (pmol)	
		+dnaA	-dnaA
Expt. I:			
M13oriC2LB5 RF	600	342	18
HaeII-digested M13oriC2LB5 RF	600	45	9
M13ΔE101 RF (vector)	600	18	9
HaeII-digested M13ΔE101 RF	600	13	6
Exp. II:			
HaeII-digested M13oriC2LB5 RF	600	45	8
HaeII fragment A (oriC)	360*	45	7
HaeII fragment B	240*	12	6

*Equivalent to 600 pmol of M13oriC2LB5 digest.

Tab. 4. Requirements for replication of a restriction fragment containing oriC.*

Component omitted	DNA synthesis (pmol)
None	61
HU protein	51
SSB	18
RNA polymerase	6
Gyrase A subunit	38
Gyrase B subunit	19
dnaB protein	8
dnaC protein	13
Primase	21
dnaA protein	15
DNA pol III holoenzyme	3

*Topoisomerase I and RNase H were present to maintain oriC template specificity and dnaA dependence. Reactions contained 360 pmol of the HaeII fragment A containing oriC.

glycol (7) may be due, in part, to a macromolecular crowding effect (21) which concentrates the DNA template as well as scarce replication enzymes. Such polymers complicate the isolation of products of the reaction, inasmuch as the DNA becomes highly catenated by the action of DNA gyrase or by the combined action of topoisomerase I and exonuclease III (22).

By systematically altering the levels of each reaction component in the absence of such polymers, an acceptable level of replication was observed with an increased level of gyrase A. In the absence of polyvinyl alcohol, a strong dependence on the gyrase A subunit was observed. As in presence of polyvinyl alcohol, replication in its absence with the increased level of gyrase A depended on dnaA protein and is specific for oriC containing DNAs. The absence of polyvinyl alcohol minimizes catenation and, as shown below, permits isolation of an intermediate in replication.

Replication can be Separated into Successive Stages of RNA Synthesis and DNA Replication

The reaction could be staged by omission of DNA polymerase III holoenzyme from an initial reaction and by addition of specific inhibitors of RNA polyermase in a second incubation. Rifampicin, which inhibits initiation, and streptolydigin, which inhibits RNA elongation, when present at the outset inhibited DNA synthesis almost entirely (17 pmol). Addition of the inhibitors after the first stage of incubation concomitant with the addition of DNA polymerase III holoenzyme permitted DNA synthesis to occur (267 pmol). Thus,

RNA polymerase forms a product during the first stage of incubation which is required for subsequent DNA synthesis to occur.

Other reaction components, except for those which confer specificity, were tested to determine whether any of them were required prior to or during the action of RNA polymerase in the first stage. Except for RNA polymerase, omission of any one of these proteins from the first stage and its addition with DNA polymerase III holoenzyme, rifampicin, and streptolydigin at the outset of the second stage of incubation did not markedly reduce the amount of subsequent DNA synthesis. Thus, these proteins are presumed to act after RNA polymerase. Attempts to isolate by gel filtration an intermediate which no longer requires RNA polymerase action indicated that several of the proteins including dnaA, dnaB, dnaC, and DNA gyrase were required in the first stage. In their absence no functional intermediate could be isolated. However, primase and DNA polymerase III holoenzyme were dispensable in the first stage. These results indicate that dnaA, dnaB, dnaC, and DNA gyrase are needed to form an intermediate sufficiently stable for gel filtration and functional for DNA replication.

SUMMARY

A crude soluble enzyme system capable of authentic oriC replication has been replaced by a purified enzyme system which specifically sustains replication of a variety of oriC-containing DNAs. This reconstituted system contains proteins which fall into 3 groups: initiation proteins responsible for transcription at oriC to prime DNA synthesis, specificity proteins which ensure that DNA synthesis is specific to the chromosomal origin, and replication proteins responsible for advancement of the replication fork. A number of significant questions may now be answered with this reconstituted enzyme system: Where are the transcripts formed by RNA polymerase for oriC replication located and how are they utilized? What enzymes and DNA sequences are required for bidirectional replication from oriC? How does binding of dnaA protein to the chromosomal origin direct initiation of DNA synthesis? Does DNA gyrase have a role in replication beyond supercoiling of DNA? How closely do the mechanisms that operate in small phage replication resemble those at the elongating replication forks in the in vitro replication from oriC? What elements control the frequency of initiation at the chromosomal origin?

ACKNOWLEDGMENTS

We mourn the loss of Jim Flynn whose sudden death on May 8, 1984, deprived us of a gifted and devoted colleague and friend. Had he presented this paper as planned, it would have included a report of his extensive studies of the organization and replication of pSC101, as well as the studies on oriC to which he contributed.

REFERENCES

1. Dixon, N.E., L.L. Bertsch, S.B. Biswas, P.M.J. Burgers, J.E. Flynn, R.S. Fuller, J.M. Kaguni, M. Kodaira, M.M. Stayton, and A. Kornberg (1983) Single-stranded phages as probes of replication mechanisms. In Mechanisms of DNA Replication and Recombination, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 10, N.R. Cozzarelli, ed. Alan R. Liss, Inc., New York, pp. 93-113.
2. Burgers, P.M.J., and A. Kornberg (1982) ATP activation of DNA polymerase III holoenzyme from Escherichia coli. II. Initiation complex: Stoichiometry and reactivity. J. Biol. Chem. 257:11474-11478.
3. Yasuda, S., and Y. Hirota (1977) Cloning and mapping of the replication origin of Escherichia coli. Proc. Natl. Acad. Sci., USA 74:5458-5462.
4. von Meyenburg, K., F. G. Hansen, L.C. Nielsen, and E. Riise (1978) Origin of replication, oriC, of the Escherichia coli chromosome on specialized transducing phages λasn. Mol. Gen. Genet. 160:287-295.
5. Zyskind, J.W., J.M. Cleary, W.S.A. Brusilow, N.E. Harding, and D.W. Smith (1983) Chromosomal replication origin from the marine bacterium Vibrio harveyi functions in Escherichia coli: oriC consensus sequence. Proc. Natl. Acad. Sci., USA 80:1164-1168.
6. Oka, A., K. Sugimoto, M. Takanami, and Y. Hirota (1980) Replication origin of the Escherichia coli K-12 chromosome: the size and structure of the minimum DNA segment carrying the information for autonomous replication. Mol. Gen. Genet. 178:9-20.
7. Fuller, R.S., J.M. Kaguni, and A. Kornberg (1981) Enzymatic replication of the origin of the Escherichia coli chromosome. Proc. Natl. Acad. Sci., USA 78:7370-7374.
8. Kaguni, J.M., R.S. Fuller, and A. Kornberg (1982) Enzymatic replication of the E. coli chromosomal origin is bidirectional. Nature (London) 296:623-627.
9. Kaguni, J.M., and A. Kornberg (1984) Replication initiated at the origin (oriC) of the E. coli chromosome reconstituted with purified enzymes. Cell, in press.
10. Hirota, Y., J. Mordoh, and F. Jacob (1970) On the process of cellular division in E. coli. III. Thermosensitive mutants of Escherichia coli altered in the process of DNA initiation. J. Mol. Biol. 53:369-387.
11. Hasunuma, K., and M. Sekiguchi (1977) Replication of plasmid pSC101 in Escherichia coli K12: requirement for dnaA function. Mol. Gen. Genet. 154:225-230.

12. Frey, J., M. Chandler, and L. Caro (1979) The effects of an Escherichia coli dnaAts mutation on the replication of the plasmids ColE1, pSC101, R100.1, and RTF-TC. Mol. Gen. Genet. 174:117-126.
13. Chakraborty, T., K. Yoshinaga, H. Lother, and W. Messer (1982) Purification of the E. coli dnaA gene product. EMBO J. 1:1545-1549.
14. Fuller, R.S., and A. Kornberg (1983) Purified dnaA protein in initiation of replication at the Escherichia coli chromosomal origin of replication. Proc. Natl. Acad. Sci., USA 80:5817-5821.
15. Fuller, R.S., B.E. Funnell, and A. Kornberg (1984) The dnaA protein complex with the E. coli chromosomal replication origin (oriC) and other DNA sites. Cell (submitted for publication).
16. Hansen, F.G., and K.V. Rasmussen (1977) Regulation of the dnaA product in Escherichia coli. Mol. Gen. Genet. 155:219-225.
17. Kaguni, J.M., and A. Kornberg (1984) Topoisomerase I confers specificity in enzymatic replication of the Escherichia coli chromosomal origin. J. Biol. Chem. 259:8578-8583.
18. Ogawa, T., G.G. Pickett, T. Kogoma, and A. Kornberg (1984) RNase H confers specificity in the dnaA-dependent initiation of replication at the unique origin of the Escherichia coli chromosome in vivo and in vitro. Proc. Natl. Acad. Sci., USA 81:1040-1044.
19. Dixon, N.E., and A. Kornberg (1984) Protein HU in the enzymatic replication of the chromosomal origin of Escherichia coli. Proc. Natl. Acad. Sci., USA 81:424-428.
20. Staudenbauer, W.L., E. Scherzinger, and E. Lanka (1979) Replication of the colicin E1 plasmid in extracts of Escherichia coli: uncoupling of leading strand from lagging strand synthesis. Mol. Gen. Genet. 177:113-120.
21. Zimmerman, S.B., and B.H. Pheiffer (1983) Macromolecular crowding allows blunt-end ligation by DNA ligases from rat liver or Escherichia coli. Proc. Natl. Acad. Sci., USA 80:5852-5856.
22. Low, R.L., J.M. Kaguni, and A. Kornberg (1984) Potent catenation of supercoiled and gapped DNA circles by topoisomerase I in the presence of a hydrophilic polymer. J. Biol. Chem. 259:4576-4581.

ORIGIN AND INITIATION SITES OF λdv DNA REPLICATION IN VITRO

Toshiki Tsurimoto, Haruhiko Kouhara, and Kenichi Matsubara

Institute for Molecular and Cellular Biology
Osaka University
Suita, Osaka 565, Japan

ABSTRACT

The sequence of λ DNA essential for the unique initiation of replication was analyzed in an in vitro replication system. Fragments of λ DNA were inserted into pBR322 and used as templates or were circularized in vitro in the absence of pBR322 and employed in the same tests. A 165-bp region left of the EcoRI site in the O gene of the λ genome was defined as the functional origin. This region, which we defined as the ori region, carries, in order, the 4 19-bp repeat sequences where the O protein binds (ori-repeats), an A+T-rich stretch, and a region that constitutes part of a large palindromic structure. Regions that have long been suspected to participate in λ DNA replication initiation, ice and oop were not required for the O, P-dependent λ-specific replication initiation. The λdv and the "ori region plus" recombinant plasmids initiated DNA synthesis at or around this region, and the reaction depended on the presence of λ-coded initiators, O and P proteins.

Early replicative intermediates of λdv were prepared in an in vitro replication system in the presence of ddCTP, an inhibitor of DNA chain elongation. This system allows only that DNA synthesis that is a result of replication initiation. Using this system, the initiation site(s) of the DNA synthesis was finely analyzed by mapping the transition sites from primer RNA to DNA synthesis. Short-chain DNAs produced from regions near the ori region were purified from the intermediates. A fraction of the short-chain DNAs was covalently linked to primer RNA. The 5'-ends that had been linked to RNA (transition sites) were exposed by alkaline hydrolysis, labeled with ^{32}P, and the transition sites were mapped along the nucleotide sequence of the genome. Two striking features emerged from this analysis: (i) The transition sites are located on both sides of the ori region, and no transition arose within the 165-bp ori region; (ii) The transition sites on both sides are not

unique, but multiple, and are clustered in one of the 2 strands. Furthermore, their orientation demonstrates that the DNA synthesis in initiation of replication from the 2 sides of the ori region converge. The frequency of the "leftward" DNA synthesis is several times higher than that of "rightward" synthesis. These results reflect asymmetric bidirectional replication of λdv DNA, and may also reflect replication of λ phage DNA.

INTRODUCTION

The initiation process of DNA replication, which controls the whole process of replication, is tightly regulated by multiple components. The key process in initiation has been intensively studied in several prokaryote replication systems, mostly in Escherichia coli, and many enzymes and various DNA signals that participate in the initiation reaction have been identified (1,2). The replication mechanism of bacteriophage λ and plasmid λdv (a minireplicon of λ phage) have also been intensively studied (for a review, see Ref. 3). A large number of genetic elements have been identified, reflecting the complicated processes that regulate the initiation of λ replication (4).

The replication initiation of λ phage or λdv plasmid depends on 2 λ-coded initiator proteins, the products of O and P genes (5). Replication initiates at or around a unique site located within the O gene (about 81% of the λ phage genetic map) and proceeds bidirectionally (6,7). Prior to the initiation, the initiation region must be "activated" by transcription (transcriptional activation; Ref. 8,9), though the mechanism of this is unknown. The replication origin has been defined by several cis-dominant replication-defective mutations (10,11), which have been mapped at or around the origin defined by electron microscopic studies. The nucleotide sequence in this region shows 3 characteristic structures around the defined origin (12,13): (i) 4 19-bp repeating sequences arranged tandemly (named ori-repeats), (ii) an A+T-rich stretch consisting of about 40 bp, (iii) a 28-bp large palindromic sequence with an EcoRI site (see Fig. 8). These structures together make up a region of about 200 bp that has been termed ori (for replication origin).

So far, however, in vivo studies have failed to show if the defined ori is the essential structure (functional origin) for replication of λ or λdv. Lusky and Hobom (14,15) have proposed that another signal, in addition to the 200-bp stretch of ori, is important for the replication of λ in vivo. They named the hypothetical functional region for replication "inceptor" (ice) and claimed that it is located far to the left of ori, in the middle of the cII gene. They further postulated that it would be a site of switching from primer RNA to DNA synthesis. No further evidence to support this idea has been obtained, however, and another line of evidence obtained by Moore et al. (16) cast doubt on the notion that ice is an indispensable site for λ replication.

Recently, biochemical studies have revealed further details of the replication mechanism. Many E. coli components participating in the elongation of DNA chains have been purified (17). In the case of λ, the initiation proteins O and P have been purified and characterized (18,19). The O protein binds specifically to the ori-repeats (20), and the P protein interacts with E. coli replication protein, dnaB (18,21). Purified dnaK protein interacts with both the O and P proteins (22). The O and P proteins were postulated to interact from a genetical study (23) but this has not been proved in in vitro studies with purified proteins. Despite the progress of interaction studies, nothing is yet known of how these proteins trigger the initiation of DNA replication.

An in vitro system has been developed that allows replication of λdv in the presence of the O and P proteins (24,25,26). This system allows de novo replication of an externally added mature λdv template, and is highly useful for analyzing the DNA signals that function in the initiation event and other molecular events associated with the initiation, such as location of initiation site(s). This paper describes the results of studies to define the functional origin of λ and map the initiation sites of replication, where the transition from primer RNA to DNA synthesis occurs.

RESULTS AND DISCUSSION

Defining the ori-Region Essential for Initiation of λdv Replication In vitro

Replication activity of pBR322-cloned λ ori fragments. We have developed an in vitro replication system for λdv DNA that depends on externally added O and P proteins (26). This system was used to analyze the minimal region of λ DNA essential for replication. As mentioned in the "Introduction," particular attention has been paid to ice and ori.

We constructed several pBR322-based recombinant plasmids carrying various regions around λ ori (Fig. 1). The replication ability of the recombinant DNAs in closed circular form was assayed in the presence or absence of O and P proteins (Tab. 1). pMY44, pOri-1, and pOA-4 carry respectively 165-bp, 354-bp, and 715-bp regions located to the left of the EcoRI site in the O gene (Fig. 1). The 165-bp region of pMY44 covers just the 3 unique structures of ori as mentioned above (see Fig. 8). The 715-bp region of pOA-4 covers ice and the oopRNA region, in addition to ori. pOri-D1 is similar to pOA-4 but lacks its rightmost portion, i.e., a 46-bp A+T-rich stretch in ori. pMY61-16 carries an additional 186-bp region to the right of pOri-1. In the absence of O and P proteins, λdv DNA does not replicate in the in vitro system. However, these pBR322-based plasmids allowed DNA synthesis to some extent, initiated from the pBR322-origin of replication. Addition of the O and P proteins

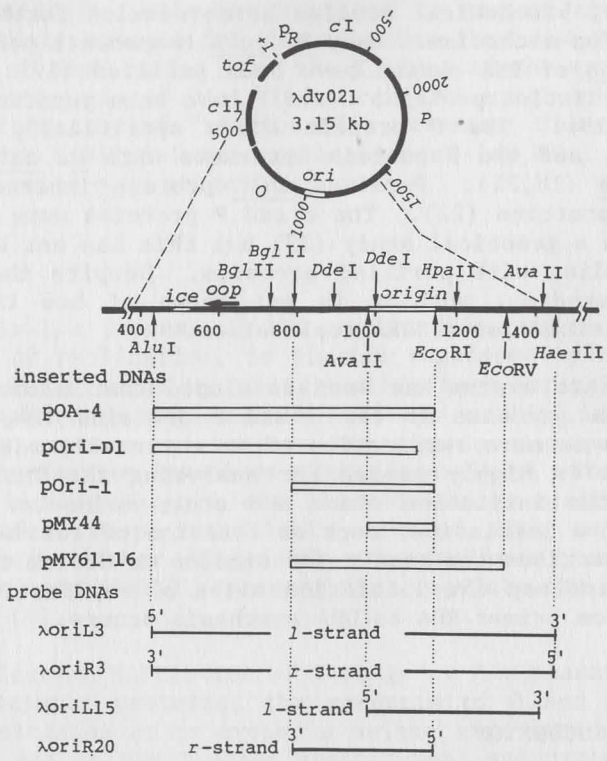


Fig. 1. λ fragments carried in the pBR322-based recombinant plasmids used as templates for the in vitro replication system. The genome of λdv021 (4,27) is shown with relevant restriction sites and the regions for ice and ori (open boxes). Genes cI, tof, cII, O, and P are shown in thick lines. oopRNA is indicated by an arrow. Numbers outside the circle indicate nucleotide numbers from mRNA starting point (+1) at p_R. The recombinant plasmids were constructed by inserting the fragments between EcoRI and BamHI sites of pBR322 in the same orientation. Constructions of pOA-4 (18), pOri-D1 (28), and pOri-1 (29) have been reported. pMY44 carries a 165-bp λ fragment isolated by AvaII (983) and EcoRI (1147) digestion. The AvaII end was converted to a BamHI end by end-filling by T4 DNA polymerase followed by ligation with BamHI linker. pMY61-16 carries the region between BglII (793) and EcoRV (1333). This plasmid was constructed by insertion of a 186-bp fragment covering EcoRI (1147) to EcoRV (1,333) into the EcoRI site of pOri-1. The EcoRV end was joined to the EcoRI end using EcoRI linker. Probe DNAs demonstrate regions of λ genome that were used, in single-stranded form, to purify short-chain DNAs (see text).

Fig. 1. (Continued)

DNAs covering these regions have been cloned into single-stranded M13 phage vectors, mp8 and mp9 (30). Lines represent the DNA regions and strands cloned in M13 phage DNAs. λoriL3 and λoriR3 carried the sequences of the l- and r-strands, respectively, covering the AluI (432) to HaeIII (1439) region. λoriL15 carried the l-strand spanning the 2 AvaII sites (983 to 1416). λoriR20 carried the r-strand of the region from BglII (793) to EcoRI (1147). The single-stranded phage DNAs were purified as described in (31), except that BND-cellulose chromatography was omitted.

stimulated DNA synthesis of pMY44, pOA-4, pOri-1, and pMY61-16, but not of pBR322 or pOri-D1. These results demonstrate that the functional origin of λ is located within the 165-bp region in pMY44. The A+T-rich stretch was indispensable for the λ replication, since pOri-D1 was inactive. All these DNAs, except for pMY61-16, lacked the 28-bp-palindromic sequence, because EcoRI truncated the palindrome. Since the activities of pMY61-16 and other active templates were the same, the complete palindromic sequence is dispensable to the replication. Whether a fraction of the palindromic sequence is needed is unknown.

Lusky and Hobom have argued that a region called ice takes part in the initiation of DNA synthesis. Our study has clearly shown that the ice region can be deleted without impairing the O,P-dependent replication ability in vitro. Recently, we have constructed a λdv genome that is completely devoid of the ice signal but which replicates autonomously (unpub. observation). Therefore, we conclude that ice is not essential for O,P-dependent λ replication, either in vitro or in vivo.

Replication of λ is sensitive to rifampicin, an inhibitor of RNA polymerase. The O,P-dependent DNA synthesis of pMY44, as well as λdv, was found to be sensitive to this drug (data not shown), showing that the minimized 165-bp λ ori sequence retained the original requirement for this enzyme. Since the 165-bp fragment lacks a normal promoter, the transcription needed for activating the ori may occur at a promoter(s) in the vector pBR322.

Replication activity of λ ori circles. In the experiments with pBR322-based recombinant plasmids, the replication activities of λ and pBR322 were superimposed. Though the results shown in Tab. 1 clearly demonstrated the presence of the functional λ origin within the 165-bp region, one could argue that it might be active only under the influence of the coexistent pBR322 replication system. To eliminate this possibility, we constructed the corresponding λ DNA fragment that had been circularized in vitro (λ ori circles). For

Tab. 1. Replication in vitro of pBR322-based recombinant plasmids carrying various fragments from around λ origin.

Plasmids	DNA synthesis (pmoles) Without O and P proteins	With O and P proteins	differences
Experiment 1.			
pOA-1	74.4	150.4	76.0
pOri-D1	80.8	89.6	8.8
pOri-1	58.4	134.4	76.0
pBR322	72.0	80.8	8.8
λdv021	1.1	248.0	246.9
Experiment 2.			
pOri-1	162.2	226.2	64.0
pMY44	145.2	241.2	96.0
pMY61-16	115.2	209.5	94.3
pBR322	148.6	155.8	7.2
λdv021	2.8	198.8	196.0

Recombinant plasmids carrying various fragments of λ (see Fig. 1) were added as templates in the in vitro replication system (26). Experiment 1 was carried out in the standard reaction mixture (25 µl) containing 40 mM Hepes (pH 8.0), 80 mM KCl, 2 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 50 µg/ml of bovine serum albumin, 100 µg/ml of creatine kinase, 21.6 mM creatine phosphate, 6 % (w/v) polyethylene glycol (PEG) 20,000, 11 mM $M_g(CH_3COO)_2$, 100 µM dNTP with 100 cpm/pmol of [α-^{32}P]dCTP, 8 µg/ml of template DNA and 120 µg of E. coli YS-1 lysate prepared according to the method of Fuller et al (32). The mixture was incubated at 30°C for 30 min with or without O (20 µg/ml) and P (16 µg/ml) proteins. Experiment 2 was carried out similarly, except that 25 µM dNTP, 10 µg/ml of DNA, 30 µg/ml of O protein, and 10 µg/ml of P protein were employed in a volume of 20 µl. The results are expressed as total dNMP incorporated. Differences between DNA syntheses with and without O and P proteins are shown in the right column. As the lot numbers of E. coli lysates used in experiments 1 and 2 were different, replication activity associated with pBR322 was somewhat higher in experiment 2 than in experiment 1.

this purpose, DNA fragments that had been cloned in pBR322 were extracted and circularized by DNA ligase under very dilute conditions in the presence of ethidium bromide to introduce negative turns in the DNA circle (Fig. 2). Since the λ ori fragments in question are too small to use in such an assay, a 0.9-kb coding region for chloramphenicol-resistance gene (Cm^r) of Tn9 (33) was employed as a space DNA which showed no replication activity. The λ fragments carried in pMY44 and pMY61-16 were first inserted into an EcoRI site located in the center of the Cm^r gene carried in pBR322. These Cm^r-λ ori fragments were extracted from the recombinant plasmids and circularized. c53X and cLDX are λ ori circles carrying 165-bp and 540-bp λ fragments within the Cm^r gene, respectively. The λ ori in each

circle was transcribed in the same orientation as in λdv from p_{lac}, which was joined with the Cm^r gene (see legend to Fig. 2). cLB, which was used as a positive control in this assay system, carried a 1.9-kb λ fragment covering a region from p_R to ori. cV2 is a circle consisting only of the spacer Cm^r gene. The replication activities of these small template DNAs were assayed similarly as before. The results in Tab. 2 demonstrate that without O and P proteins, none of the small templates initiated DNA replication. In the presence of these proteins, cLB, c53X, and cLDX, but not cV2, initiated DNA synthesis. Thus, the in vitro assay system allows replication of minimized λ ori circles, in the absence of any influence from the replication of coexistent pBR322. The results for the small templates were in complete agreement with those obtained with pBR322-based recombinant plasmids. The 165-bp λ fragment (c53X), active in the O,P-dependent DNA replication, showed the same level of DNA synthesis as cLB and cLDX demonstrating that the 165-bp fragment carries all the signals necessary for the O,P-dependent DNA synthesis in the in vitro system. Thus, we defined the 165-bp region as the "ori region", indicating that it is the minimal functional origin of λ replication.

Identifying the Region Where λ DNA Replication is Initiated.

The initiation site of DNA replication on λdv021 and pMY44 was investigated using ddCTP, which blocks chain elongation at an early phase of replication (26). The template DNAs were replicated in the presence of [α-^{32}P]dCTP and ddCTP, and specific activity of the ^{32}P-count in each restriction fragment was analyzed (Fig. 3). Those fragments located in the O gene were found to be preferentially labeled when λdv021 was used as a template (Fig. 3A). Because DNA elongation is halted randomly by ddCTP, the relative specific activity in each fragment should reflect its distance from the initiation site of DNA synthesis, which, in this case, must lie within, or close to, the ori region. The gradual decrease of radioactivity on both sides of the ori region demonstrates that the DNA synthesis proceeds bidirectionally. However, the bidirectional synthesis seems to be asymmetric, with leftward synthesis stronger than rightward.

pMY44 was analyzed similarly. The result in Fig. 3B shows again that the initiation of DNA synthesis occurs within, or close to, the ori region. Asymmetric DNA synthesis was again observed, but in this case rightward synthesis was stronger. The cause of this difference between λdv and pMY44 is unknown. It could reflect a difference between the sequences flanking the ori region, which either promote or inhibit procession of the replication fork, or it could reflect a difference between transcription proceeding either across or away from the ori region in connection with primer RNA synthesis or transcriptional activation.

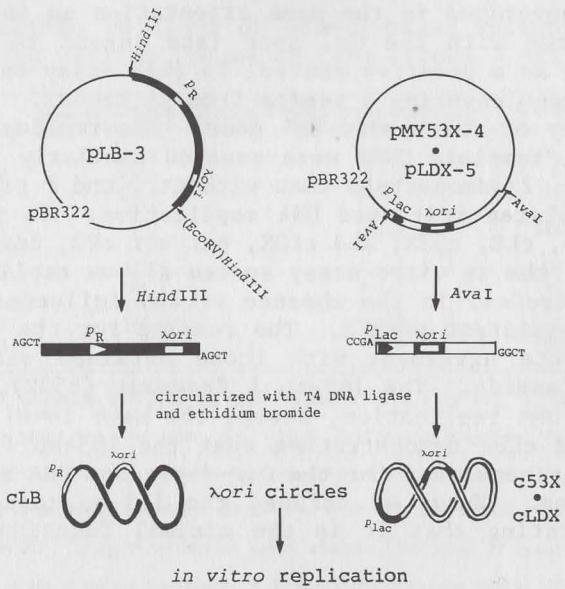


Fig. 2. Construction of λ ori circles. pLB-3 is a pBR322-based recombinant plasmid, carrying a 1.9-kb fragment of λdv covering from HindIII (-563) to EcoRV (1,333) inserted into the HindIII site of the vector. The EcoRV end had been converted to a HindIII end by ligation with HindIII linker. Thin and thick lines represent, respectively, regions of pBR322 and λdv sequence. The open arrow and box in the λ sequence are P_R (a right λ promoter) and λ ori region, respectively. pMY53X-4 and pLDX-5 are pBR322-based recombinant plasmids that carry λ fragments inserted into the Cm^r gene used as spacer (open bar). The spacer DNA, a 0.9 kb fragment consisting of a lactose operon promoter (plac: filled arrow) joined with 0.8 kb TaqI fragment coding for Cm^r gene of Tn9 (details will be described elsewhere), was inserted into the AvaI site of pBR322. The 165-bp and 540-bp λ fragments carried in pMY44 and pMY61-16, respectively, were inserted into a unique EcoRI site in the spacer DNA, resulting in pMY53X-4 and pLDX-5. The ori region in the inserted λ fragment was transcribed from the plac in the same direction as in λdv genome, where transcription is initiated at P_R.

pLB-3 was digested with HindIII, and pMY53X-4 and pLDX-5 were digested with AvaI. DNA fragments of 1.9 kb from pLB-3, 1.2 kb from pMY53X-4 and 1.5 kb from pLDX-5 were purified by electrophoresis and self-ligated at a concentration of 1-2 μg DNA/ml in the presence of 5 μg/ml of ethidium bromide. The ligated DNA was extracted with

Fig. 2. (Continued)

phenol to remove ligase and ethidium bromide, and the resulting circles, cLB (from pLB-3), c53X (from pMY53X-4) and cLDX (from pLDX-5) were precipitated with ethanol. The majority of the DNAs in these preparations were in monomeric negatively twisted closed circular form.

The result in Fig. 3B alone does not allow us to conclude that the DNA synthesis of pMY44 is bidirectional. However, our mapping studies of the transition sites from primer RNA to DNA synthesis indicate that the DNA synthesis of this plasmid also proceeds bidirectionally, with several transition sites located on both strands around the ori region (data not shown).

Determination of the Multiple Initiation Sites Flanking the ori Region

Analysis of transition sites from primer RNA to DNA synthesis. To study the first step in the initiation of λ DNA synthesis, we attempted to localize the site(s) of transition from primer RNA synthesis to DNA synthesis. For this purpose, λdv021 DNA was added as a template to the in vitro replication system. The reaction conditions were essentially the same as described in Tab. 1, except that 300 μl of reaction mixture contained 20 μg/ml of λdv DNA, 20 μM of each dNTP with 4.2 mCi/μmol of [methyl-^{3}H]TTP, and 40 μM ddCTP, and was incubated at 30°C for 10 min. A higher ddCTP/dCTP ratio (40/20) was chosen in this experiment than that adopted in Fig. 3 (100/100), in order to minimize the possibility of excessive chain

Tab. 2. Replication of λ ori circles in vitro.

λ ori circles	DNA synthesis (pmoles)	
	without O and P proteins	with O and P proteins
cLB	3.6	80.4
c53X	6.0	67.6
cLDX	4.4	63.6
cV2	4.4	10.8

Various fragments of λ from around ori were joined with the CM^r gene and made into closed circles in an in vitro ligation system (see text, and Fig. 2). The reactions were carried out at 30°C for 30 min in standard reaction mixture (20 μl) as described in Table 1, with 10 μg/ml of template λ ori circles. The results are expressed as total dNMP incorporated. Under the condition used, 117 pmols of dNMP was incoporated into λdv021. cV2 is the control, a 0.9kb circle consisting solely of spacer CM^r DNA.

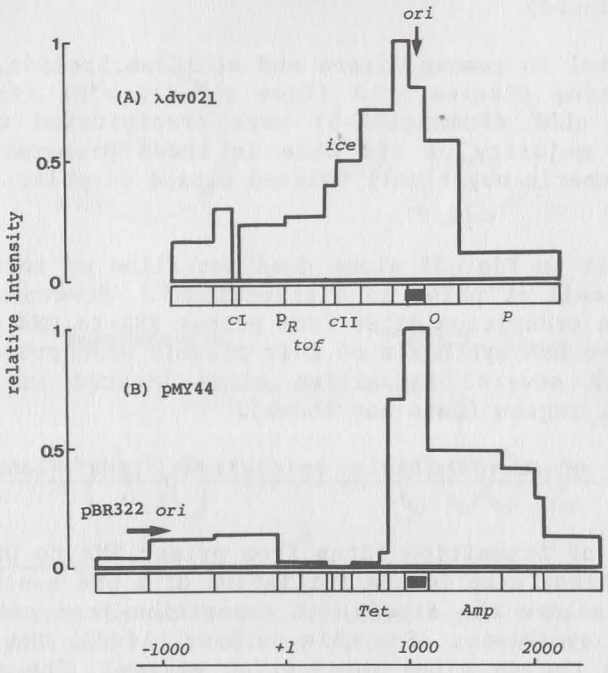


Fig. 3. Analysis of initiation sites of DNA replication in vitro in λdv021 and pMY44 genomes using ddCTP. (A) λdv021 DNA was replicated in the standard reaction mixture containing 100 μM of [α-^{32}P]dCTP and 100 μM ddCTP. The replicated DNA was purified and digested with DdeI and EcoRI. Distribution of newly incorporated radioactivity among the restriction fragments was analyzed by electrophoresis and autoradiography. The specific radioactivity of each fragment was measured by scanning with a densitometer and mapped along λdv genome (abscissa). The results are expressed as relative intensities (ordinate). The ori region is shown as a filled box in the map. For ori and ice, see text. (B) pMY44 DNA was employed similarly, except that 20 μM [α-^{32}P]dCTP and 40 μM ddCTP were used. The DNA was digested with HaeIII and analyzed by electrophoresis as before. Amp and Tet are the drug-resistance markers in pBR322. pBR322 origin and arrow are the replication origin of the vector and its direction of replication. The direction of λ ori region (filled box) in B is the same as in A. Numbers under the graphs represent nucleotide distance on λdv021 genome as shown in Fig. 1.

elongation that might lead to synthesis of new Okazaki fragments, i.e., general chain elongation. During this incubation, approximately 30% of the template DNAs initiated DNA synthesis (the amount

of active templates was approximately 0.9 pmols), and short-chain DNAs of 60-160 nucleotides were synthesized (corresponding to approximately 1.9 pmols). Therefore, about 2 short-chain DNAs were formed per active template. The reaction was stopped and the replicative intermediates of λdv were purified by successive chromatographies on sepharose 2B and benzoylated-naphthoylated DEAE cellulose (31). Details of replicative intermediate purification will be described elsewhere.

The replicative intermediates were heated at 95°C for 3 min., and denatured short-piece DNA was purified with a sepharose CL-4B column. Fractions with kd values of 0.4 to 0.8, which contained 60-160 nucleotide short-piece DNAs, were pooled. Their 5' ends were masked with T4 polynucleotide kinase and 0.1 mM ATP at 37°C for 30 min., then the sample was incubated in 0.15 M NaOH and 4 mM EDTA at 37°C for 14 hr. to expose 5'-OH groups that had been covalently linked with primer RNA. The freshly exposed 5'-OH groups were specifically labeled with T4 polynucleotide kinase and 1.3 μM [γ-^{32}P]ATP at 0°C for 60 min. (34). Approximately 10% of total short-pieces were end-labeled. Note that this figure represents the fraction of DNA pieces that had been linked to primer RNA. The remaining 90% of the fragments had been freed from the primer RNA before or during sample preparation.

As shown in Fig. 3, when the chain terminator ddCTP was administered, practically all the DNA synthesis occurred in or around the ori region. Two cycles of hybridization with appropriate probes were carried out as shown below, to select those fragments carrying only the sequences of the ori region and its vicinity. Possible contamination by Okazaki fragments representing chain elongation that might have occurred in other regions of the plasmid DNA was eliminated by these hybridizations. Thus, the junction between primer RNA and short DNA in our sample can be taken to represent the transition site(s) in the earliest phases of replication.

The labeled DNA was divided into 2 equal portions, (I) and (II), which were mixed respectively with an excess (30 μg) of λoriL3 DNA and λoriR3 DNA (see Fig. 1). The DNA in each sample was denatured and renatured at 65°C for 6-14 hr. (first hybridization). The nonrenatured, end-labeled short DNA chains were purified by chromatography using CL-4B, and fractions with kd values of 0.4-0.8 were collected. Short pieces for l- or r-strand synthesis were enriched in these fractions. To these samples from (I) (II), was added, respectively, an excess (30 μg) of λoriR20 or λoriL15 (see Fig. 1), and denaturing and renaturing treatments were done as above (second hybridization). The renatured DNA was purified by applying the sample on a CL-4B column and collecting the DNAs in the excluded fractions. The labeled materials in (I) and (II) represent short-chain DNAs of l- and r-strands, respectively, at or around the λ-ori region.

The hybrids of the short-chain DNAs and probe DNAs were digested with S1 nuclease to remove single-stranded regions (35), and the mixture was passed through Sephadex G-100 columns. The excluded DNAs were pooled and digested with appropriate restriction enzymes. Through these series of treatments, about 3.0% and 10% of the initially incorporated ^{32}P counts ertr recovered in (I) and (II), respectively. The digested DNAs were analyzed by electrophoresis in denaturing gel.

Transition sites are located outside of the ori region. The S1-treated sample (I) was digested with EcoRI and electrophoresed. This experiment was designed to detect l-strand DNA that had undergone transition within the ori region, since such DNA would show a band(s) shorter than 165 nucleotides. However, no discrete bands were detected (Fig. 4, lane 1). The same sample exhibited several bands upon treatment with AvaII (lane 2), indicating that several transition sites are located to the left of the ori region. Further analysis of these sites will be described below.

We then examined the S1-treated sample (II) by digestion with DdeI to detect the transition sites on the r-strand within the ori region. A band of 152-155 nucleotides appeared (Fig. 4, lane 3), indicating that a transition site is located at around the 1155 position, to the right of the ori region (marked A in Fig. 7 and 8). Some shorter bands were also observed. These bands appeared reproducibly, irrespective of whether the sample had been treated with DdeI or HpaII (lanes 3 and 4), or restriction enzyme treatment omitted (data not shown). Thus, they must be "background" bands of this assay system, representing preferential termination products that tend to terminate in cytosine clusters in the ori-repeats in the presence of ddCTP (see legend to Fig. 6). Thus, we conclude that all the transition sites are located outside of the ori region. The HpaII digest exhibited additional bands representing other transition sites to the right of the ori region (see below).

To locate the transition sites outside the ori region, the S1-treated l-strand specific sample (I) was digested with DdeI or AvaII. The results in Fig. 5 demonstrate that transition sites are located to the left of the ori region, in a stretch from 793 to 1,000. Many bands of various intensities were observed in both samples. Since the region analyzed carries 2 DdeI sites (887 and 1,000), transition sites were determined precisely with the AvaII digested sample, and 3 major clusters of transition sites (Z, Y, and V) were identified within the region from 895 to 980. Z is composed of one strong and 2 weak bands; Y, of 2 strong bands; and V, of 4 strong and 7 weak bands. In addition, 5 clusters of weak bands (x, w, s, t, u) were noted. "s" and "x" overlap in lane 1.

Transition sites of the r-strand located to the right of the ori region were analyzed from DNAs in sample (II) that had been

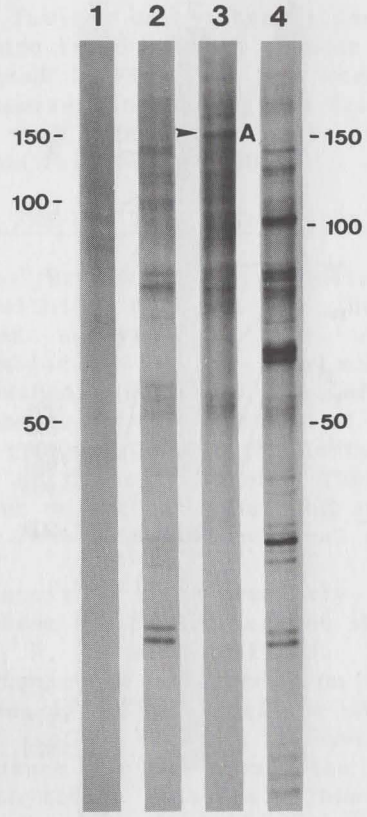


Fig. 4. Search for transition sites within or close to the ori region. Purified, end-labeled l-strand short-chain DNA that had been hybridized with λoriR20 followed by S1-digestion was digested with EcoRI (lane 1) or AvaII (lane 2). Purified, end-labeled short-chain DNA that had been hybridized with λoriL15 followed by S1-digestion was digested with DdeI (lane 3) and HpaII (lane 4). Numbers beside the figures represent the nucleotide length.

end-labeled and hybridized with λoriL15. The sample was digested with EcoRI or HpaII (Fig. 6). Transition sites between 1150 and 1416 can be located by this analysis. Seven major clusters (A, B, E, F, G, H, I) of transition sites were observed. Each cluster appeared in ladders of 4-8 bands. Weaker transition sites (c, d, j, k, l) were also visible. Weak ladders between G and H clusters and between H and I clusters (lanes 2 and 3) are part of the "background" that was also observed in Fig. 4 (for the background, see above, also legend to Fig. 6).

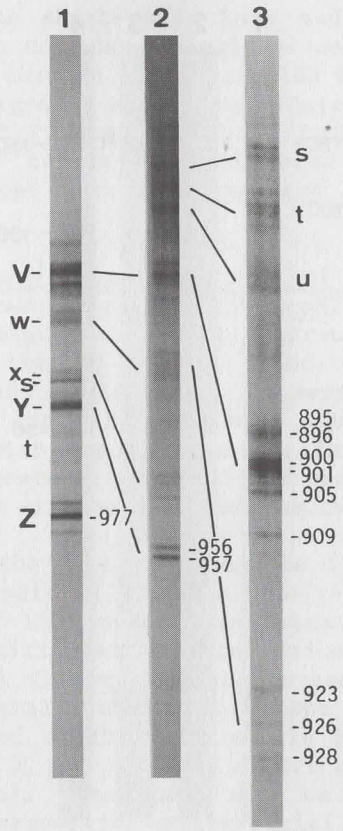


Fig. 5. Analysis of l-strand transition sites located to the left of the ori region. Purified, end-labeled l-strand short-chain DNA, hybridized with λoriR20 and treated with S1 nuclease, was digested with DdeI (lane 1) and AvaII (lanes 2 and 3). The data are assembled from 3 independent electrophoreses. Lane 3 is the result of long electrophoresis of the same sample as in lane 2. Z, Y, and V are the major transition clusters that consist of strong bands and some flanking weak bands. x, w, u, t, and s are the minor transition sites. Corresponding bands are connected by lines. Numbers indicate the locations of the 5' ends of the DNA pieces along the nucleotide sequence of λ (see Fig. 7 and 8). These locations were determined from the positions of restriction enzyme sites and the measured nucleotide lengths of bands obtained by comparing with size markers.

Next, the transition sites were examined in a wider stretch of DNA covering about 1 kb around the ori region, using λoriL3 and

λoriR3 as probes. No further strong transition sites were detected, although weak sites were found on both strands (data not shown: the results are incorporated in Fig. 7). The weak transition sites in the l-strand were dispersed in the regions from 750 to 800 and from 1230 to 1310. The weak transition sites in the r-strand were clustered in the region from 910 to 930.

A Summary Map Showing RNA-DNA Transition Sites in λdv Replication

The foregoing analyses by electrophoresis enabled us to locate the transition sites within 2 nucleotides. The relative intensities of the major bands were assayed in 2 or 3 independent experiments. The results are assembled in Fig. 7. Most of the transitions for the rightward DNA synthesis occur to the left of the ori region, while minor transitions occur to the right of the ori region. Conversely, most of the transitions for the leftward DNA synthesis are located to the right of the ori region. Therefore, the major DNA chain initiations occur on both sides of the ori region and the syntheses converge. The result is bidirectional replication.

To locate the transition sites precisely, the ^{32}P-labeled mononucleotides obtained from the 5' ends of the short DNA chains (V, Y, Z and A, B, E, F, G, H, I) were analyzed. The results (data not shown) agreed well with those obtained from the analyses of chain length. Figure 8 summarizes the transition sites on the nucleotide sequence of the λ ori region. So far, we have not been able to detect a consensus sequence for the transition sites, except for the preferential use of thymidine residues. Thus, 12 out of 19 strong transition sites were T, and only 4, 2, and 1 were A, C, and G, respectively.

No transition sites were discovered within the ori region. This implies that the ori region essential for replication initiation contains no site of initiation of primer RNA synthesis. Two possibilities can be considered concerning the synthesis of primer RNA. First, the primer RNA synthesis might be initiated at multiple sites that are somehow associated with the transition sites we observed. Each of the product RNAs would then be short molecules. Alternatively, the primer RNA might be made as a large transcript starting far upstream of the transition sites. Rightward RNA synthesis from p_R is one candidate for the long right primer. However, there is no known long leftward RNA synthesis that goes across the ori region. Therefore, the leftward long primer must be made by a new mechanism; and this, of course, might also operate for rightward primer synthesis. These problems can only be solved by analysis of the 5' ends of the primer RNAs.

Our observations also demonstrate that the ori region does not contain the site(s) of transition from primer RNA to DNA. At present, too little information is available to link these observations with the fact that O protein binds the ori-repeating sequences (20).

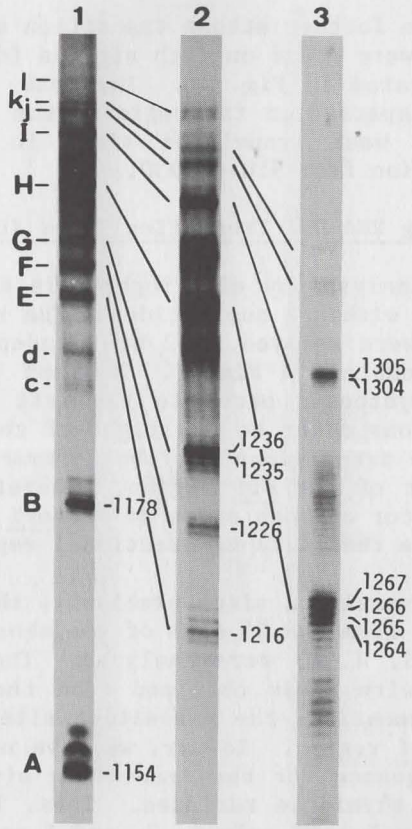


Fig. 6. Analysis of transition sites on r-strand to the right of the ori region. Purified, end-labeled r-strand short-chain DNA, hybridized with λoriL15 and treated with S1 nuclease, was digested with EcoRI (lane 1) or HpaII (lane 2). Lane 3 is the result of long electrophoresis of the same sample as in lane 2. Clusters of major transition sites are indicated as A, B, E, F, G, H, and I, and minor sites are indicated as c, d, j, k, and l. The HpaII digest (lanes 2 and 3 in b) shows 2 additional ladders which consist of background bands produced as a result of preferential chain termination of DNA synthesis of ddCTP at the cytosine cluster. Ladders between H and I, and between G and H may represent the DNA chains initiated respectively at sites B and A and terminated at the cytosine cluster at positions 1,092-1,096. We have three reasons to assume this: (i) The chain lengths of these bands, 57-61, and 76-84 nucleotides, match the distances from sites B and A to the termination. (ii) Nucleotides at the 5' ends of these bands are mainly C and T, suggesting that they share the same ends with the A and B bands,

Fig. 6. (Continued)

respectively (see Fig. 4). (iii) These bands did not appear in the sample digested with EcoRI, which cuts just downstream of the sites A and B. But they appeared at the same positions upon digestion with several other enzymes.

Several lines of evidence suggest that the O protein interacts with P protein (23), which in turn interacts with such host-coded proteins as dnaB (18,21) and dnaK (22). The ori sequence may thus be used as a signal that attracts the replication machinery complex. This complex could alter the structure of neighboring DNA regions to facilitate initiation of primer RNA synthesis. Alternatively, they could facilitate modification or processing of the incoming primer RNA molecules or could regulate their synthesis.

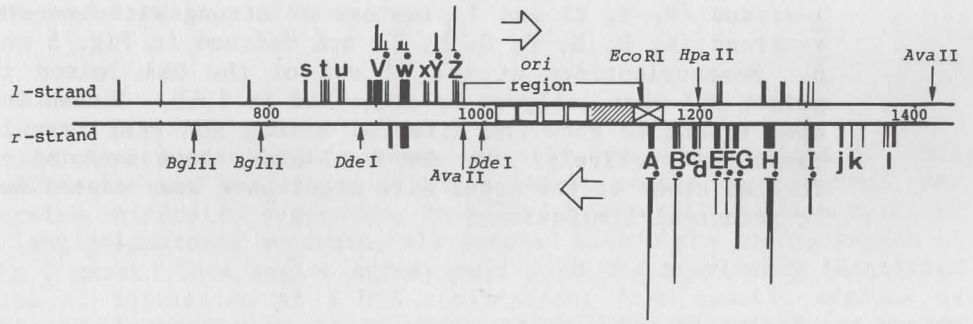


Fig. 7. Summary map of the transition sites on l- and r-strands of λ genome. Vertical bars on each strand indicate locations of the transition sites observed in this work. The letters correspond to those in Fig. 5 and 6. Additional bars above and below the asterisked letters are drawn to represent relative frequency of occurrence of the transition. These were obtained from densitomeric tracing of the autoradiographed bands. Results for minor bands that could not be accurately measured are not shown. The results for l- and r-strands can be compared with each other, since they were taken from the same autoradiography using ^{32}P-labeled DNAs that were prepared from 2 identical aliquots prepared for each strand (not shown). Arrows represent the direction of DNA synthesis. The ori region carries ori-repeats (4 boxes), an A+T-rich stretch (////), along with a part of a palindromic 28-bp sequence (⋈). The cutting sites of restriction enzyme used in the analyses are also shown. It is possible that some weaker transition sites have been missed in these analyses.

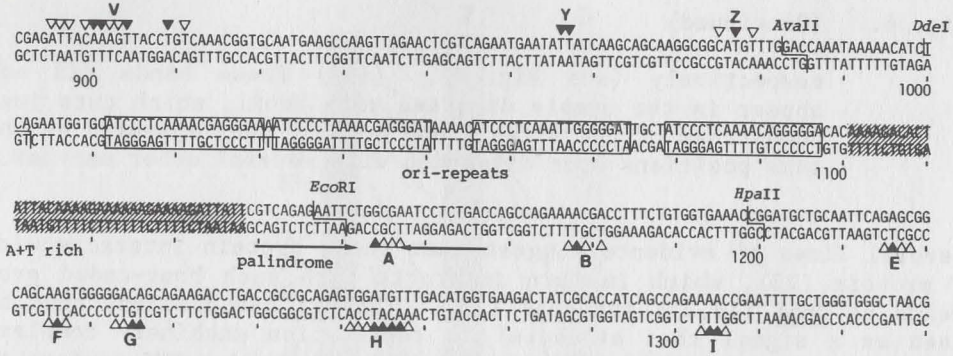


Fig. 8. Transition sites as represented in the nucleotide sequence of λ around the ori region. λ DNA sequence from 891 to 1,330 is shown. The "ori region" is the region from AvaII (983) to EcoRI (1,147). Ori-repeats (boxed), A+T-rich stretch (///), and palindromic sequence (arrows) are shown. Three clusters of strong transition sites on the l-strand (V, Y, Z) and 7 clusters of strong sites on the r-strand (A, B, E, F, G, H, I) are defined in Fig. 5 and 6. Mononucleotides at the 5' end of the DNA joined to primer RNA were analyzed as described in (36). Closed and open triangles show the sites of strong and weak transitions, respectively. In the A cluster, some weak sites located close to the EcoRI site might have been missed due to technical limitations.

Of the leftward and rightward DNA syntheses, the former is several times more frequent. This asymmetry was reflected in the higher recovery of r-strand than l-strand after S1 treatment. This fact agrees well with the results of Fig. 3, which show that leftward DNA synthesis occurs more strongly than rightward DNA synthesis in the process of bidirectional DNA replication of λdv in vitro. The implications of this asymmetry are unknown, but it could reflect a difference in the initiation mechanisms for leftward and rightward DNA synthesis.

The multiplicity of transition sites from primer RNA to DNA synthesis in λdv replication is in sharp contrast with the situation in ColE1 (37,38), which synthesizes a long primer RNA starting at a unique site, followed by transition at a unique site. This difference means that the "multiple site" mechanism must be different from that of the "unique site" mechanism observed with ColE1. It would be of interest to see, at least in procaryotes, how the "multiple site" and "unique site" mechanisms are distributed among different replicons.

Although multiple, the λ transition sites seem not to be distributed randomly: in fact, the replicon seems to have favored sites that appear reproducibly in independent experiments, although how these sites are selected is unclear. At this stage, we can only infer that combinations of local nucleotide sequences and higher order structures of the DNA are responsible for the selection of the transition sites, and that thymidine residues play an important role. The possibility remains that different transition sites might be selected when the experimental system is modified.

Since the in vitro system used exhibits a similar mode of DNA synthesis to that observed in in vivo replication, in the requirements for the reaction, the bidirectional nature of the replication, and the ori-dependent initiation, the initiation process of λ replication in vivo may also show transition at multiple sites. However, the possibility cannot be ruled out that the finding of multiple transition sites reflects a lack of a factor(s) necessary for selection of a unique site(s). In this connection, it will be of interest to analyze the in vivo transition sites of λ DNA replication.

CONCLUSION

Deletion studies have shown that the functional origin of λ DNA replication can be defined within a 165-bp region (ori region) that carries reiterated sequences, an A+T-rich stretch, and a portion of a long palindromic sequence, all located within the coding region of the O gene. This region agrees well with the previously identified site of initiation of λ DNA replication, from genetic studies as well as electron microscopic examinations. The minimized ori region replicates only in the presence of O and P proteins and the RNA polymerase-dependent RNA synthesis. The DNA synthesis that is promoted by the ori region, as examined by incorporation tests, proceeds bidirectionally.

Short-chain DNAs joined to primer RNAs were purified from earliest replicative intermediates of λdv, as prepared in an O,P-dependent in vitro replication system. The 5' ends of these short DNA chains (i.e., the transition sites from primer RNA to DNA synthesis) were freed from RNA, specifically labeled with ^{32}P, then mapped along the known nucleotide sequence of λ. The results showed that transition occurred at multiple sites located on both strands flanking the λ ori region. The orientation of the transition sites demonstrates that the chain elongation proceeds convergently. This, again, is in accord with the bidirectional nature of initiation in λ replication. The fact that no transition sites were observed within the ori region implies that the sequence essential for λ replication involves neither the transition sites from primer RNA to DNA nor the initiation sites of primer RNA synthesis. To understand the initia-

tion mechanism of λ replication totally, it is necessary to elucidate the sites and mechanisms of primer RNA synthesis.

ACKNOWLEDGMENTS

We are grateful to Dr. Asao. Fujiyama for his valuable advice throughout this work. This work was supported by Scientific Research Grants from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Tomizawa, J.-I., and G. Selzer (1979) Initiation of DNA synthesis in Escherichia coli. Ann. Rev. Biochem. 48:999-1034.
2. Nossal, N.G. (1983) Prokaryotic DNA replication systems. Ann. Rev. Biochem. 53:581-615.
3. Matsubara, K. (1981) Replication control system in lambda dv. Plasmid 5:32-52.
4. Tsurimoto, T., and K. Matsubara (1983) Replication of bacteriophage λ DNA. Cold Spring Harbor Symp. Quant. Biol. 47:681-691.
5. Ogawa, T., and J. Tomizawa (1968) Replication of bacteriophage DNA. I. Replication of DNA of lambda phage defective in early function. J. Mol. Biol. 38:217-225.
6. Schnös, M., and R.B. Inman (1970) The position of branch point in replicating lambda DNA. J. Mol. Biol. 51:61-73.
7. Inman, R.B. (1981) DNA replication. In Proceedings of the 3rd Cleveland Symposium on Macromolecules, A.G. Walton, ed. Elsevier Publishing Co., Amsterdam, pp. 155-165.
8. Dove, W., E. Hargrove, M. Ohashi, F. Haugli, and A. Guha (1969) Replicator activation in lambda. Japan J. Genet. Vol. 44, Suppl. 1, pp. 11-22.
9. Furth, M.E., W.F. Dove, and B.J. Meyer (1982) Specificity determinants for bacteriophage λ DNA replication. III. Activation of replication in λric mutants by transcription outside of ori. J. Mol. Biol. 154:65-83.
10. Dove, W.F., H. Inokuchi, and W.F. Stevens (1971) Replication control in phage lambda. In The Bacteriophage Lambda, A.D. Hershey, Cold Spring Harbor Laboratory, New York, pp. 747-771.
11. Rambach, A. (1973) Replicator mutants of bacteriophage λ: Characterization of 2 subclasses. Virology 54:270-277.
12. Denniston-Thompson, K., D.D. Moore, K.E. Kruger, M.E. Furth, and F.R. Blattner (1977) Physical structure of replication origin of bacteriophage lambda. Science 198:1051-1056.
13. Grosschedl, R., and G. Hobom (1979) DNA sequence and structural homologies of the replication origins of lambdoid bacteriophages. Nature 277:621-627.
14. Lusky, M., and G. Hobom (1979) Inceptor and origin of DNA replication in lambdoid coliphages. I. The λ DNA minimal replication system. Gene 6:137-172.

15. Lusky, M., and G. Hobom (1979) Inceptor and origin of replication in lambdoid coliphages. II. The λ DNA maximal replication system. Gene 6:173-197.
16. Moore, D.D., K. Denniston-Thompson, K.E. Kruger, M.E. Furth, B.G. Williams, D.L. Daniels, and F.R. Blattner (1979) Dissection and comparative anatomy of the origins of replication of lambdoid phages. Cold Spring Harbor Symp. Quant. Biol. 43:155-163.
17. Kornberg, A. (1979) Aspects of DNA replication. Cold Spring Harbor Symp. Quant. Biol. 43:1-9.
18. Tsurimoto, T., T. Hase, H. Matsubara, and K. Matsubara (1982) Bacteriophage lambda initiator: Preparation from a strain that overproduces the O and P proteins. Mol. Gen. Genet. 187:79-86.
19. Roberts, J.D. and R. McMacken (1983) The bacteriophage λ O replication protein: Isolation and characterization of the amplified initiator. Nucl. Acids Res. 11:7435-7452.
20. Tsurmioto, T., and K. Matsubara (1981) Purified bacteriophage λ O protein binds to four repeating sequences at the λ replication origin. Nucl. Acids Res. 9:1798-1799.
21. Wickner, S.H. (1979) DNA replication proteins of Escherichia coli and phage λ. Cold Spring Harbor Symp. Quant. Biol. 43:303-310.
22. Zylicz, M., J.H. LeBowitz, R. McMacken, and C. Georgopoulos (1983) The dnaK protein of Escherichia coli possesses as ATPase and autophosphorylating activity and is essential in an in vitro DNA replication system. Proc. Natl. Acad. Sci., USA 80:6431-6435.
23. Tomizawa, J. (1971) Functional cooperation of genes O and P. In The Bacteriophage Lambda, A. Hershey, ed. Cold Spring Harbor Laboratory, New York, pp. 549-552.
24. Anderl, A., and A. Klein (1982) Replication of λdv DNA in vitro. Nucl. Acids Res. 10:1733-1740.
25. Wold, M.S., J.B. Mallory, J.D. Roberts, J.H. LeBowitz, and R. McMacken (1982) Initiation of bacteriophage λ DNA replication in vitro with purified λ replication proteins. Proc. Natl. Acad. Sci., USA 79:6176-6180.
26. Tsurimoto, T., and K. Matsubara (1982) Replication of λdv plasmid in vitro promoted by purified λ O and P proteins. Proc. Natl. Acad. Sci., USA 79:7639-7643.
27. Chow, L.T., N. Davidson, and D. Berg (1974) Electron microscopic study of the structure of λdv DNAs. J. Mol. Biol. 86:69-89.
28. Tsurimoto, T., and K. Matsubara (1981) Purification of bacteriophage λ O protein that specifically binds to the origin of replication. Mol. Gen. Genet. 181:325-331.
29. Tsurimoto, T., and K. Matsubara (1981) Interaction of bacteriophage lambda O protein with the lambda origin sequence. In The Initiation of DNA Replication, ICN-UCLA Symp. 22, D.S. Ray, ed. Academic Press, New York, pp. 263-275.

30. Messing, J., and J. Vieira (1982) A new pair of M13 vectors for selecting either DNA strand of double-digested restriction fragments. Gene 19:269-276.
31. Hay, R.T., and M.L. DePamphilis (1982) Initiation of SV40 DNA replication in vivo: Location and structure of 5' ends of DNA synthesized in the ori region. Cell 28:767-779.
32. Fuller, R.S., J.M. Kaguni, and A. Kornberg (1981) Enzymatic replication of the origin of the E. coli chromosome. Proc. Natl. Acad. Sci, USA 78:7370-7374.
33. Alton, N.K., and D. Vapnek (1979) Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature 282:864-869.
34. Okazaki, R., S. Hirose, T. Okazaki, T. Ogawa, and Y. Kurosawa (1975) Assay of RNA-linked nascent DNA pieces with polynucleotide kinase. Biochem. Biophys. Res. Commun. 62:1018-1024.
35. Fujiyama, A., Y. Kohara, and T. Okazaki (1981) Initiation sites for discontinuous DNA synthesis of bacteriophage T7. Proc. Natl. Acad. Sci., USA 78:903-907.
36. Ogawa, T., and T. Okazaki (1979) RNA-linked nascent DNA pieces in phage T7-infected Escherichia coli. III. Detection of intact primer RNA. Nucl. Acids Res. 7:1621-1633.
37. Tomizawa, J-I., H. Ohmori, and R.E. Bird (1977) Origin of replication of colicin E1 plasmid DNA. Proc. Natl. Acad. Sci., USA 74:1865-1869.
38. Itoh, T., and J-I. Tomizawa (1980) Formation of an RNA primer for initiation of replication of colE1 DNA by ribonuclease H. Proc. Natl. Acad. Sci, USA 77:2450-2454.

BROAD HOST-RANGE PLASMID R1162: REPLICATION, INCOMPATIBILITY, AND COPY-NUMBER CONTROL

Richard J. Meyer, Lung-Shen Lin,
Kyunghoon Kim, and Michael A. Brasch

The University of Texas at Austin
Department of Microbiology
Experimental Science Building, 226
Austin, Texas 78712-1095

INTRODUCTION

R1162 is a small [8.7 kilobases (kb)]plasmid encoding resistance to sulfonamides (Su^R) and streptomycin (Sm^R), and replicating with a high copy-number in Escherichia coli (at least 10 copies per chromosome) (1). The plasmid is not self-transmissible, but may be readily mobilized by certain other conjugative plasmids, such as those of the P-1 incompatibility group (2). Originally isolated from Pseudomonas aeruginosa (3), R1162 has a broad host-range (1,4-6). Plasmids which appear very similar to R1162 have been isolated from diverse bacteria (1,7); many of these may be identical representatives of the same far-ranging plasmid. At present there do not seem to be any clear differences between R1162 and the best-studied member of this group, RSF1010 (from E. coli) (1).

Genetic Organization of R1162

Figure 1 is a map of R1162 showing cleavage sites for several restriction enzymes, and also the approximate locations of coding sequences for known plasmid functions. The Su^R and Sm^R genes are cotranscribed, with Su^R promoter proximal (6,8). The single EcoRI cleavage site lies between the structural genes for Su^R and Sm^R, and insertion of DNA into this site can have a variable effect on expression of both drug resistances. Thus, a promoter cloned in one direction can activate Sm^R, and change the level of this resistance (Ref. 6 and unpub. observations), whereas in the other direction it can reduce the level of Su^R by transcriptional interference. These characteristics are useful for molecular cloning (for example, see Ref. 9).

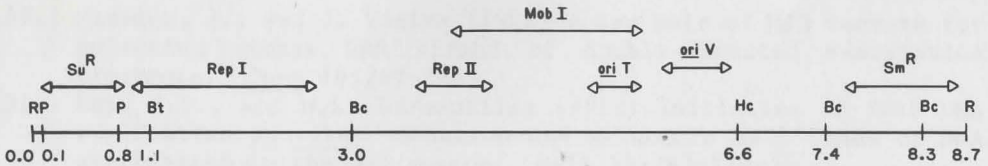


Fig. 1. Genetic organization of R1162. The cleavage sites for a few restriction enzymes are shown; numbers refer to distances, in kilobase pairs (kb), from the single EcoRI cleavage site. Abbreviations: Bc: BclI, Bt: BstEII, Hc: HincII, P: PstI, and R: EcoRI. Other abbreviations are defined in the text.

Two regions of the plasmid supply products necessary in trans for plasmid maintenance: sequences between 0.8 and 3.0 kb, and 3.8 and 4.3 kb (Rep I, II: Fig. 1). Initially, we believed that the latter DNA was dispensible, because pMS14, which lacks this DNA, can replicate (2). However, we have found that it does so by virtue of a second, low-copy number R1162 derivative, also present in the cell, which supplies RepII. Satellite plasmids consisting of oriV must have RepI and RepII functions supplied in order to be maintained in the cell.

DNA-encoding functions necessary in trans for conjugal mobilization of R1162 (Mob) are located between 3.9 and 5.7 kb. The origin for conjugal mobilization (oriT) and the origin for DNA replication (oriV), are located in the region 5.3-6.6 kb (2). The close physical association of oriT and oriV has raised the possibility of a functional interdependence (10). However, it is clear from cloning studies described below that both are capable of independent activity. No additional DNA required for replication or transfer is located to the right of the 6.6 kb position on the map.

To examine the RepI region in greater detail, we have cloned the 0.0 to 3.0 kb region containing RepI into pBR322, and generated deletions rightward and leftward into this DNA by Bal31 digestion (Fig. 2). We asked whether the resulting plasmid derivatives still contained the complete RepI region by testing their ability to support replication of a satellite plasmid, pMS125, which is RepII$^+$ oriV$^+$ but deleted for RepI. The results for some derivatives are shown in Fig. 2. Essential sequences are evidently located as far left as 1.07 kb, and as far right as 2.57 kb. We also tested whether any of the cloned DNAs would complement pMS88, a derivative of R1162 which is temperature-sensitive for maintenance due to mutation in RepI (2). The plasmids unable to provide RepI function for pMS125 were also unable to complement pMS88. The simplest interpretation is that there is a single transcriptional unit lying within the region 0.91-2.63 kb, which may encode one or more proteins.

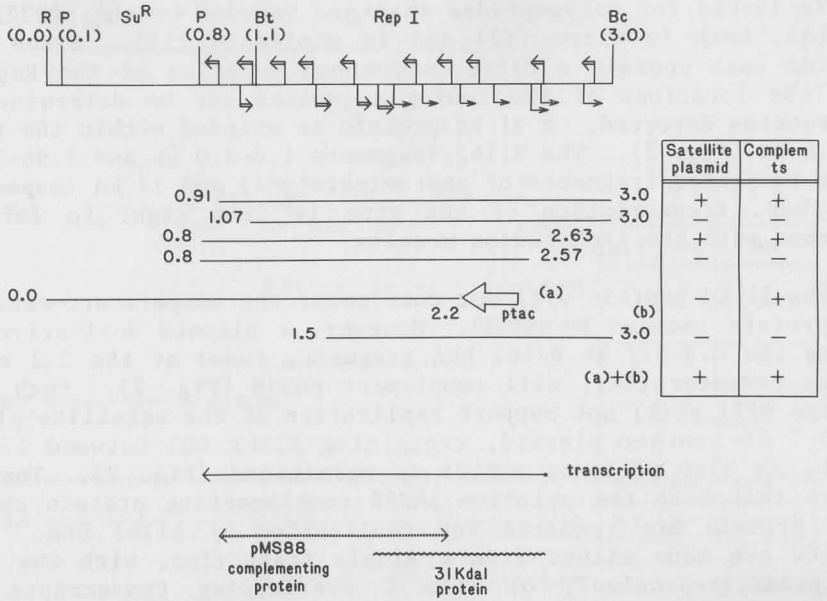


Fig. 2. Region of R1162 containing genes required in trans for plasmid maintenance. A physical map with some restriction enzyme cleavage sites (see Fig. 1) is at the top. The vertical lines indicate the endpoints of Bal31-generated deletions, and the attached arrows the direction of transcription within R1162 tested at that point. An open arrow means that transcription was detected. Below, fragments of R1162 DNA cloned into pBR322 are represented by the horizontal lines. Whether each fragment is able to support replication of an oriV$^+$ RepII$^+$ satellite plasmid, or to complement the R1162 derivative pMS88, which has a mutation in RepI, is shown at the right. At the bottom of the figure, the location of a gene encoding a 31 kd protein, and also the location and maximum size of DNA encoding the product complementing the defect in pMS88 are indicated; the direction of transcription of these genes is shown just above.

We fused the variable ends of the Bal31 deletions to a lacZ fragment lacking both the lacZ promoter and amino-terminal coding sequence (11). The resulting fusions can be used to determine the direction of transcription within R1162 DNA at each junction point. This is possible even when the fusions are out of phase, since the assay for β-galactosidase is very sensitive (11). The results are shown in Fig 2: the arrows indicate the direction of transcription being tested; open arrows mean that β-galactosidase is produced. There is transcription from right to left throughout the RepI region, and also transcription rightward in the left segment.

We looked for polypeptides that are encoded by the pBR322-RepI plasmids, both in vitro (12) and in minicells (13). Since these plasmids each contain a different, known deletion of the RepI region, the locations of the coding sequences can be determined for any proteins detected. A 31 kd protein is encoded within the region 1.7-2.6 kb (Fig. 2). The R1162 fragments 1.8-3.0 kb and 1.96-3.0 kb encode truncated fragments of approximately 29 and 22 kd respectively: thus, transcription of the gene is from right to left, in agreement with the lacZ fusion results.

The 31 kd protein will not complement the temperature-sensitive RepI protein encoded by pMS88. However, a plasmid derivative containing the 0.0-2.2 kb R1162 DNA fragment, fused at the 2.2 end to the tac promoter (14), will complement pMS88 (Fig. 2). Such a derivative will still not support replication of the satellite plasmid pMS125. If another plasmid, containing R1162 DNA between 1.5 and 3.0 kb, is also present, pMS125 is maintained (Fig. 2). Thus, it appears that both the putative pMS88 complementing protein and the 31 kd protein are required for replication of R1162 DNA. These products are made either from a single transcript, with the 31 kd gene promoter-proximal, or from 2 overlapping transcripts with closely spaced promoters. We have not yet clearly identified a protein corresponding to the pMS88 complementing activity. However, Scherzinger et al. (15) have shown that at least 2 proteins, required for replication of RSF1010 DNA in vitro, are encoded in the region which we call RepI. They have purified a 27 kd polypeptide which, from their mapping studies, is probably the pMS88-complementing protein. If this is so, the 1.07-2.57 kb RepI region is probably just sufficient to encode 27 and 31 kd polypeptides without overlap.

Genetic Organization of the oriT-OriV Region

We have used molecular cloning methods to characterize more fully the region of R1162 containing oriT and oriV. Some of the results of this analysis are given in Fig. 3. Specific fragments of DNA from R1162 were cloned into pBR322, and then tested for an active oriV as outlined in Fig. 4. Because the cloned R1162 DNA lacked the Rep functions, we tested whether this DNA could replicate as a satellite plasmid when Rep functions were provided in trans. Cloned R1162 DNA linked to DNA encoding kanamycin-resistance (Km^R) was released from cloning vector DNA by SalI digestion, and used to transform MV10 (R1162) to Km^R (Fig. 4). Plasmid DNA, extracted from transformants resistant to kanamycin but sensitive to carbenicillin (Cb^S) was then used to transform MV10 (pMS69) and MV10 (pBR322) to Km^R. The plasmid pMS69 is a chimera of pBR322 and an R1162 derivative that contains all Rep functions (2). If a $Km^R(Cb^S)$ satellite plasmid had been present in the transformed MV10 (R1162) cells, then the Km^R transformants of MV10 (pMS69) would not, in general, also be Sm^R, since pMS69 could provide any missing functions for plasmid maintenance. In contrast, the Km^R transformants of MV10 (pBR322)

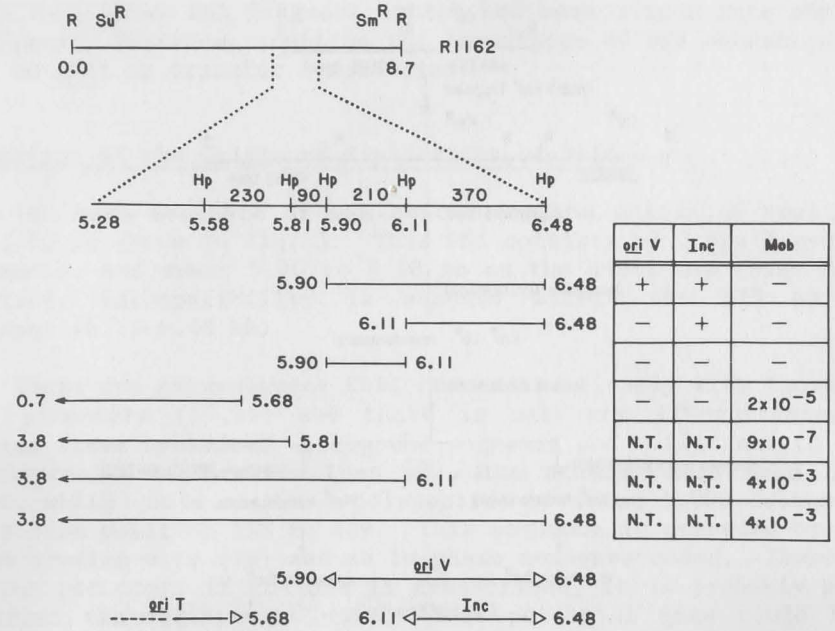


Fig. 3. The oriV region of R1162. R1162 DNA between 5.28 and 6.48 kb (Fig. 1), containing the origin of plasmid replication, is expanded and the location of several HpaII (Hp) cleavage sites are shown. Numbers between the HpaII cleavage sites give fragment sizes in base pairs (bp). Below are represented various fragments of DNA cloned into pBR322, and, to the right, the results for testing each of these for ability to form satellite plasmid (oriV), expression of incompatibility (Inc), and conjugal mobilization by R751 (Mob). If the plasmid is mobilizable, the frequency (transconjugants per donor cell) is given.

would all be Sm^R, since cotransformation of R1162 would be required for maintenance of the satellite. If the initial $Km^R(Cb^S)$ transformants arose by a mechanism not involving satellite plasmid formation (e.g., by deletion of the Cb^R gene in pBR322, or by insertion of the Km^R gene into R1162 by recombination), then this pattern of acquiring Sm^R in the second transformation would not be observed (Fig. 4).

We found that the $oriV^+$ function required the presence of 2 contiguous HpaII fragments of 210 and 370 bp (Fig. 3). Neither fragment by itself was capable of replication as a satellite plasmid. Different fragments of R1162 DNA, cloned into pBR322, were also tested for expression of incompatibility. DNA of each plasmid derivative was used to transform MV10 (R1162), and the resulting Cb^R

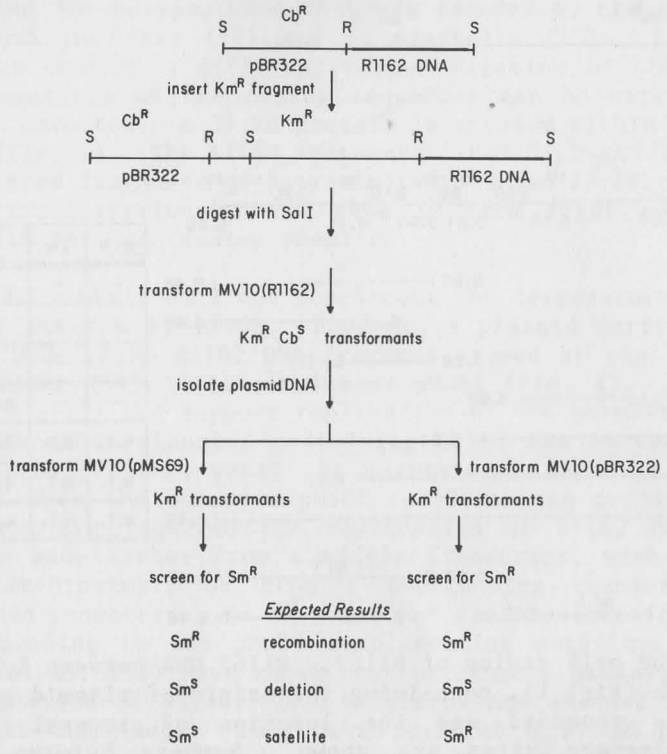


Fig. 4. Method for detection of satellite plasmids. The procedure is outlined in the text.

cells screened for retention of R1162 by scoring for Sm^R. The incompatibility determinant was localized to the 370 bp HpaII fragment. Thus, the incompatibility determinant and oriV are closely linked in R1162 (16).

We previously observed that pMS94, a pBR322 derivative containing R1162 DNA between 0.70 and 5.68 kb, was mobilizable by the self-transmissible IncP1 plasmid R751 (2). Thus, the oriV-Inc DNA lying between 5.90 and 6.48 kb is not required for mobilization, although oriV and oriT are closely linked on R1162 (2). However, we noticed that the mobilization frequency of pMS94 was typically about $2X10^{-5}$ transconjugants per donor cell, under conditions where R1162 was mobilized at a frequency of 0.1 (Fig. 3). It is possible, therefore, that sequences lying between oriV and oriT affect the efficiency of mobilization. We measured the mobilization frequencies for pBR322 derivatives containing R1162 DNA from 3.8 kb to a variable endpoint within the oriV region. In each case, the R1162 DNA was located in an identical position and orientation within pBR322. The results suggest that DNA sequences in the oriV region are important for mobilization. The mobilization, at intermediate frequencies, of

the 0.70-5.68 bp DNA fragment, which had been cloned into pBR322 at a different location, confirms the importance of DNA sequences adjacent to oriT on transfer frequencies.

Properties of the Origin of Replication of R1162

The base sequence of DNA containing the origin of replication of R1162 is shown in Fig. 5. This DNA consists of 2 HpaII-generated fragments, and spans 5.90 to 6.48 kb on the R1162 map (Fig. 3). In addition, incompatibility is encoded within the 370 bp HpaII fragment (6.11-6.48 kb).

There are no sequences that correspond closely with those of E. coli promoters (17,18) and there is only one ATG-initiated open reading frame contained within the sequence and sufficient to encode a polypeptide of greater than 40 amino acids. This open reading frame, which could encode a polypeptide of about 5,000 daltons, extends from position 558 to 409. This sequence is preceded by a ribosome binding site (19) and an in-phase nonsense codon. There is no obvious promoter; if the DNA is transcribed, it is probably part of a larger transcriptional unit. This potential gene could be expressed in the cloned DNA, with transcription provided by an exogenous promoter. However, the putative polypeptide is not required for expression of incompatibility or an active oriV. We deleted 150 bp of DNA, from the RsaI cleavage site (position 435) to the end of the 370 bp fragment (position 587). The remaining DNA (positions 1-435) was able to form a satellite plasmid, and also continued to express incompatibility. This does not exclude a regulatory role for the polypeptide.

The result with the RsaI-generated deletion means that the incompatibility determinant must lie between positions 212 and 435. The outstanding feature of this region is the presence of 3 perfectly conserved direct repeats, part of which is repeated a fourth time (boxed sequences, Fig. 5). In view of the results with other plasmids (20-23), it is likely that these direct repeats are necessary for expression of incompatibility and copy-number control. This is considered further in the next section. Finally, the adjacent 210 bp HpaII fragment shows extensive 2-fold symmetry (Fig. 6).

Both the 210 and 370 bp HpaII fragments are required in cis for an active oriV (although at least 150 bp of the latter fragment are dispensible). We find that neither fragment will replicate as a satellite plasmid in a cell containing (a) pMS94 (Rep functions provided but other HpaII fragment missing); (b) R1162 (Rep functions and other HpaII fragment provided in trans as part of replicon); or (c) pMS94 plus cloned other fragment (Rep functions and other HpaII fragment provided in trans but not as part of active replicon). However, the 2 fragments need not be contiguous, or in the same relative orientation. The plasmids pMS320 and pMS321, which contain

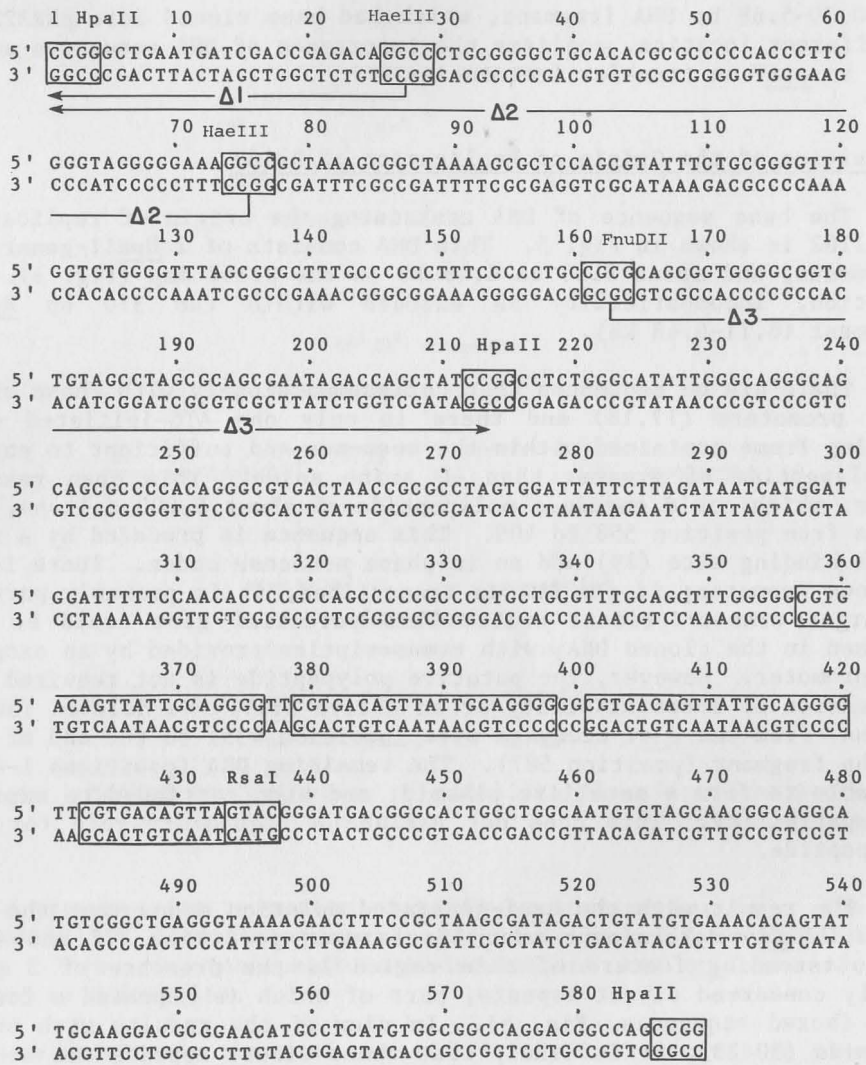


Fig. 5. Base sequence of DNA containing the origin of replication and determinant for incompatibility of R1162. The recognition sequences for certain restriction enzymes are boxed, as are those sequences that make up the direct repeats. The locations of deletions Δ1, Δ2, and Δ3 are shown.

R1162 DNA organized as shown in Fig. 7, are both able to replicate as satellite plasmids. We have also generated in vitro deletions within the 210 bp HpaII fragment to determine which regions are essential (Fig. 5 and 6). The deletion Δ1 does not interfere with the

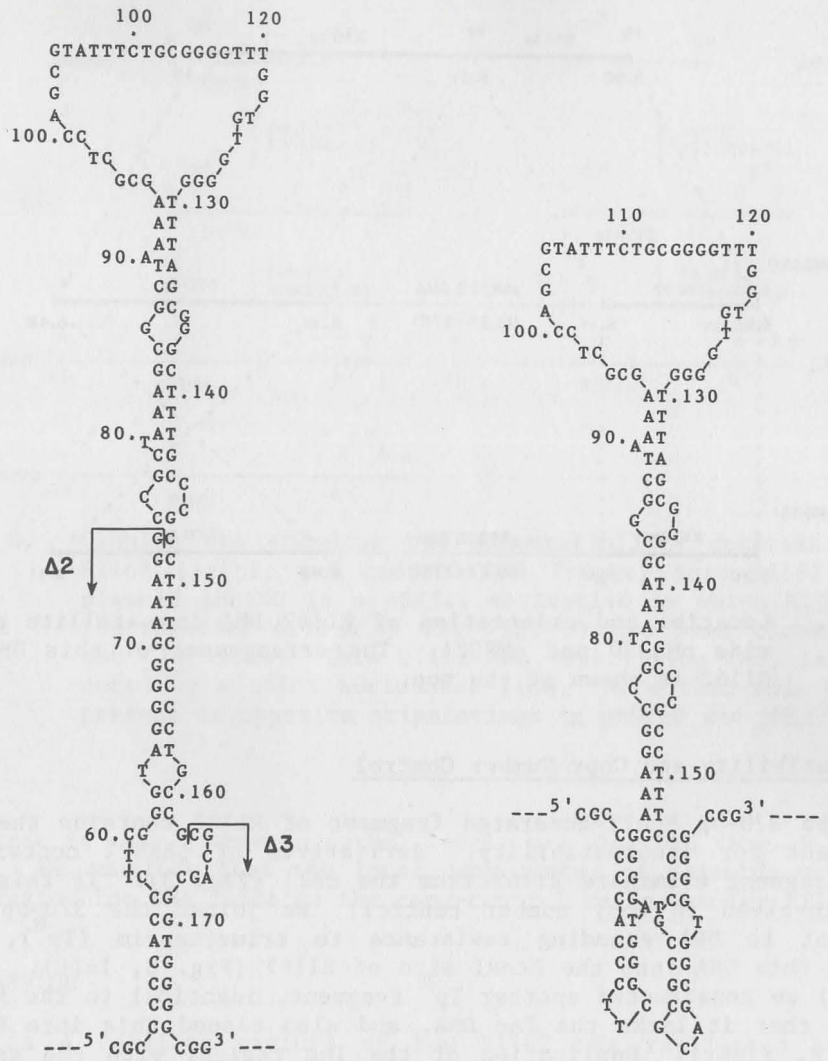


Fig. 6. Part of the base sequence of one strand of the DNA in Fig. 5, redrawn to emphasize the 2-fold symmetry. The starting points of deletions Δ2 and Δ3 are indicated.

ability of the oriV DNA to replicate as a satellite, whereas satellite plasmids cannot be obtained from DNA containing deletions Δ2 or Δ3 (Fig. 5 and 6). These results are consistent with the idea that the 2-fold symmetry of the 210-bp fragment is significant with respect to plasmid DNA replication.

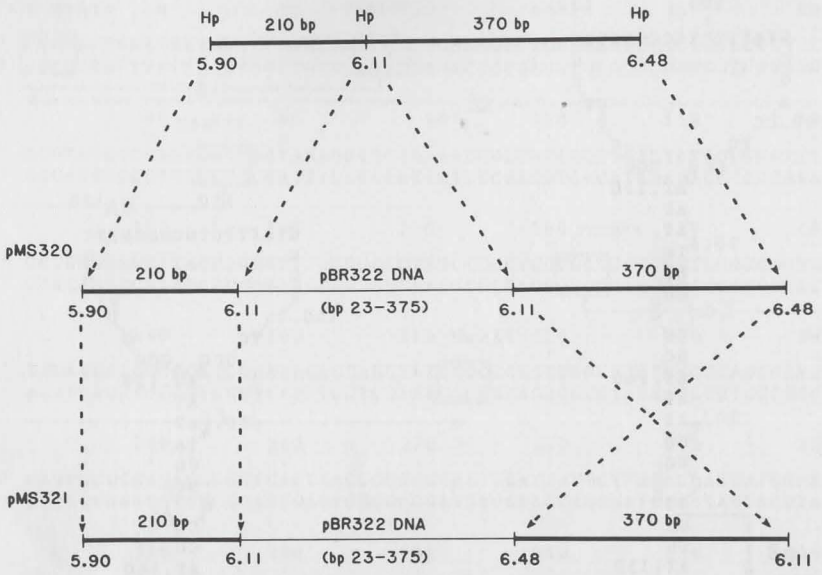


Fig. 7. Location and orientation of R1162 DNA in satellite plasmids pMS320 and pMS321. The arrangement of this DNA in R1162 is shown at the top.

Incompatibility and Copy-Number Control

The 370-bp HpaII-generated fragment of R1162 contains the determinant for incompatibility: derivatives of pBR322 containing this fragment eliminate R1162 from the cell (Fig. 3). Is this DNA also involved in copy number control? We joined the 370-bp Inc fragment to DNA encoding resistance to trimethoprim (Tp^R), and cloned this DNA into the EcoRI site of R1162 (Fig. 8, left). As a control we constructed another Tp^R fragment, identical to the first except that it lacks the Inc DNA, and also cloned this into R1162 (Fig. 8, right). Duplication of the Inc region, with the second copy at a novel location, resulted in a lower plasmid copy number (Fig. 9, lanes 4-6). The orientation of insertion of the DNA was not important. This decrease in copy number could come about because of duplication of a DNA sequence encoding a trans-acting inhibitor of plasmid replication. Alternatively, a positively acting product may be removed by binding to the DNA, perhaps at the directly repeated sequences within this DNA (Fig. 5) (2). If a constitutively produced, required plasmid product were not present in excess, then nonproductive binding to a second site could result in lower plasmid copy number, yet the product itself might not be involved in normal regulation of copy number. To exclude this possibility, we introduced the pBR322 derivative pMS94, which encodes the necessary gene products active in trans for replication of R1162, into strains containing R1162 with the duplicated Inc segment. No

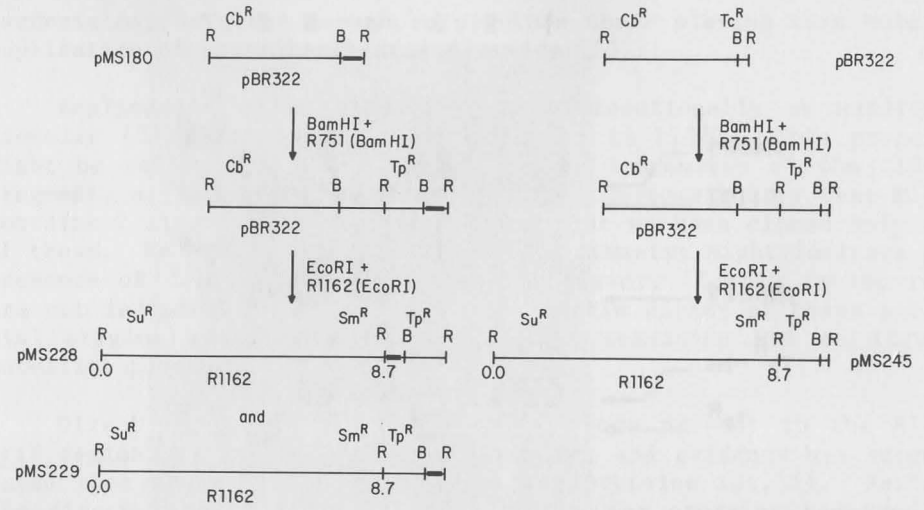


Fig. 8. Cloning DNA encoding the incompatibility determinant of R1162 (left), and control DNA (right) into R1162. The plasmid pMS180 is a pBR322 derivative in which R1162 DNA (coordinates 6.11-6.48 kb, Fig. 3) has been cloned into the ClaI site. This R1162 DNA, specifying Inc, is indicated by a thick horizontal line. Note that this DNA is present in opposite orientations in pMS228 and pMS229.

increase in copy number to normal levels was observed (Fig. 9, lanes 1-3). We conclude that the lower copy number of plasmids with the duplicated Inc DNA reflects the copy-control mechanism for R1162.

DISCUSSION

As a detailed picture of how R1162 DNA replicates becomes available, it will provide insight into how this plasmid achieves its broad host range. Several observations can already be made. First, it is clear that a large fraction of R1162 DNA encodes genes required for plasmid maintenance (Fig. 1). Moreover, 3 essential proteins, active in an in vitro system for plasmid DNA replication, have been identified by Scherzinger et al. for RSF1010 (15). In principle, broad host range can be the consequence of a plasmid providing many of its own requirements for replication or, alternatively, it can be due to universalized sites on its DNA, able to parasitize whatever host replicative functions are available. R1162 may have evolved in the former direction. Interestingly, another broad host-range plasmid, RK2, might require only one plasmid-encoded protein for replication (24,25), although other proteins are necessary for cellular maintenance (26,27).

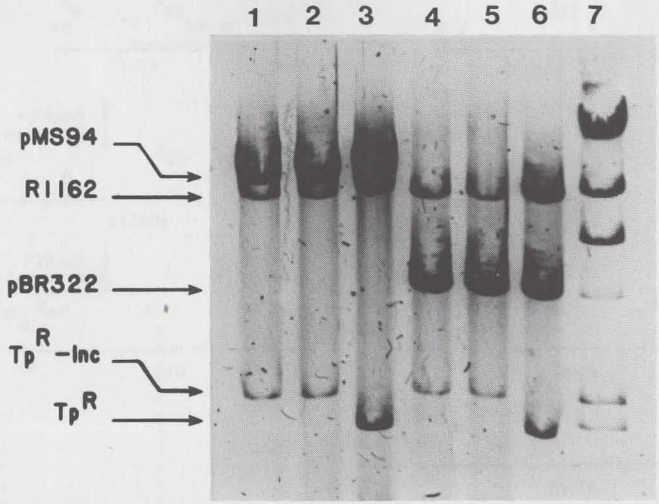


Fig. 9. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs from strains containing plasmids (see Fig. 8): Lane 1 pMS94 + pMS228, lane 2 pMS94 + pMS229, lane 3 pMS94 + pMS245, lane 4 pBR322 + pMS228, lane 5 pBR322 + pM229, lane 6 pBR322 + pMS245. Lane 7 is λ DNA, HindIII-digested.

The origin of replication of R1162 is located between 5.90 and 6.48 kb on the R1162 map, and this is quite distant from those genes providing the proteins which presumably act on it. A similar situation is observed for RK2; in contrast, for many plasmids, coding sequences sufficient for replication lie close together (28-31). Broad host-range plasmids could arise by fusion of separate replicons, followed by a slow process of homogenization: loss of redundant genetic elements along with mutation of others to broader activity. The lack of clustering of coding sequences essential for replication could reflect this evolutionary history.

The origin region of R1162 contains no open reading frames of substantial size likely to encode protein. The amount of transcriptional activity within the oriV region cannot be determined from the sequence alone. There are no putative promoters with substantial homology to both the -35 and -10 consensus sequences for the promoters of E. coli (17,18), although the promoters of broad host-range plasmids might be expected to diverge from this consensus. However, if RNA polymerase is involved in replication, it probably does not provide a primer, since replication of RSF1010 DNA can take place in vitro in the presence of rifampicin (32). It has been suggested that differences among the RNA polymerases of gram-negative

bacteria may be great enough to preclude their playing this role in replication of broad host-range plasmids (15).

Replication of RSF1010 proceeds bidirectionally or unidirectionally (33), and probably similarly so in R1162. This property might be reflected in the extensive 2-fold symmetry of the 210-bp fragment, although we have not ruled out the possibility that R1162 contains 2 closely linked origins, and that we have cloned only one of these. We considered that the 2-fold symmetry might indicate the presence of 2 origins, back-to-back. However, if this is so, they are not independent, since a deletion within either of these potential origins results in failure of the remaining DNA to form a satellite plasmid.

Direct repeats about the size of those present in the R1162 oriV region are found in several plasmids, and evidence has accumulated that these bind positively acting proteins (34,35). Perhaps the direct repeats serve as entry points for proteins required in formation of a plasmid DNA replication complex. The direct repeats are also associated with expression of incompatibility (20-23). This has led Tsutsui et al. to propose a model for control of copy number in which a positively acting product, present in constant amount in the cell, is titrated by dummy sites represented by some or all of the directly repeated sequences (20). Our results are consistent with this model, since increasing the dosages of the genes for required plasmid products does not affect the copy number of derivatives containing a duplicated Inc segment. However, the possibility of synthesis of an inhibitor, directed by the Inc DNA, has not been ruled out.

A comparison of the base sequence of the direct repeats of R1162 with those of other plasmids shows that they bear the greatest homology with those of another broad host-range plasmid, RK2 (Fig. 10) (23). Perhaps the direct repeats serve to bind host-encoded products, and the homology represents evolution toward a consensus sequence able to function in large variety of hosts. It is also conceivable that R1162 and RK2 shared a common ancestor.

Not all the sequences required in cis for replication of R1162 need be in their wild-type location or orientation with respect to

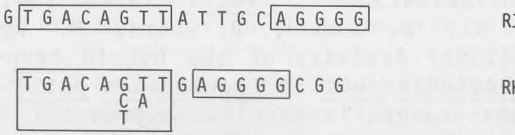


Fig. 10. Comparison of the direct repeats of R1162 and RK2 (23). The homologous regions are boxed. The sequence of one strand is shown, written 5' to 3'.

each other. This indicates that the oriV region is made up of a number of discrete functional domains, separated by nonessential segments. Possibly, replication components are assembled at one location, and then, in a primosome-like fashion, migrate along the DNA to the origin, where DNA synthesis is initiated (36).

ACKNOWLEDGMENTS

Parts of this work have been supported by the National Science Foundation (Grants PCM-8002628 and PCM-8219916), and by the Robert A. Welch Foundation (Grant F-964).

REFERENCES

1. Barth, P., and N. Grinter (1974) Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bact. 120:618-630.
2. Meyer, R., M. Hinds, and M. Brasch (1982) Properties of R1162, a broad host-range, high-copy number plasmid. J. Bact. 150:552-562.
3. Bryan, L.E., H.M. van den Elzen, and J.T. Tseng (1972) Transferable drug resistance in Pseudomonas aeruginosa. Antimicrob. Ag. Chemother. 1:22-29.
4. Gautier, F., and R. Bonewald (1980) The use of plasmid R1162 and derivatives for gene cloning in the methanol-utilizing Pseudomonas Aml. Mol. Gen. Genet. 178:375-380.
5. Quivey, Jr., R.G., R.J. Meyer, and F.R. Tabita (1981) Plasmid transfer into Rhodospirillum rubrum. Eighty-first Ann. Meeting, American Society for Microbiology (Abstract H113).
6. Meyer, R., R. Laux, G. Boch, M. Hinds, R. Bayley, and J.A. Shapiro (1982) Broad host-range Inc P-4 plasmid R1162: Effects of deletions and insertions on plasmid maintenance and host range. J. Bact. 152:140-150.
7. Grinter, N.J., and P. Barth (1976) Characterization of SmSu plasmids by restriction endonuclease cleavage and compatibility testing. J. Bact. 128:394-400.
8. Rubens, C., F. Heffron, and S. Falkow (1976) Transposition of a plasmid dexoyribonucleic acid sequence that mediates ampicillin resistance: Independence from host rec functions and orientation of insertion. J. Bact. 128:425-434.
9. Bagdasarian, M., E. Amann, R. Lurz, B. Ruckert, and M. Bagdasarian (1983) Activity of the hybrid tryp-lac (tac) promoter of Escherichia coli in Pseudomonas putida. Construction of broad host-range, controlled-expression vectors. Gene 26:273-282.
10. Nordheim, A., T. Hashimoto-Gotoh, and K.N. Timmis (1980) Location of two relaxation nick sites in R6K and single sites in pSC101 and RSF1010 close to origins of vegetative replication: Implications for conjugal transfer of plasmid deoxyribonucleic acid. J. Bact. 144:923-932.
11. Casadaban, M.J., J. Chou, and S.N. Cohen (1980) In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: Escherichia coli plasmid vectors for the detection and cloning of translational initiation signals. J. Bact. 143:971-980.
12. Pratt, J.M., G.J. Boulnois, V. Darby, E. Orr, E. Wahla, and I.B. Holland (1981) Identification of gene products programmed by restriction endonuclease DNA fragments using an E. coli in vitro system. Nucl. Acids. Res. 9:4459-4474.
13. Frazer, A.C., and R. Curtiss, III (1973) Derepression of anthranilate synthetase in purified minicells of Escherichia coli containing the Col-trp plasmid. J. Bact. 115:615-622.
14. Amann, E., J. Brosius, and M. Ptashne (1983) Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in Escherichia coli. II Gene 25:167-178.
15. Scherzinger, E., M.M. Bagdasarian, P. Scholz, R. Lurz, B. Ruckert, and M. Bagdasarian (1984) Replication of the broad host-range plasmid RSF1010: Requirement for three plasmid-encoded proteins. Proc. Natl. Acad. Sci., USA 81:654-658.
16. Lin, L.-S., and R.J. Meyer (1984) Nucleotide sequence and functional properties of DNA encoding incompatibility in the broad host-range plasmid R1162. Mol. Gen. Genet. (194:423-431).
17. Rosenberg, M. and D. Court (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. Ann. Rev. Genet. 13:319-353.
18. Hawley, D.K., and W.R. McClure (1983) Compilation and analysis of Escherichia coli promoter DNA sequences. Nucl. Acids Res. 11:2237-2255.
19. Shine, J., and K. Dalgarno (1974) The 3' terminal sequence of E. coli 16S rRNA: Complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci., USA 71:1342-1346.
20. Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsubara (1983) Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bact. 155:337-344.
21. Kamio, Y., and Y. Terawaki (1983) Nucleotide sequence of an incompatibility region of mini-Rtsl that contains five direct repeats. J. Bact. 155:1185-1191.
22. Tolun, A., and D.R. Helinski (1981) Direct repeats of the F plasmid incC region express F incompatibility. Cell 24:687-694.
23. Stalker, D.M., C.M. Thomas, and D.R. Helinski (1981) Nucleotide sequence of the region of the origin of replication of the broad host-range plasmid RK2. Mol. Gen. Genet. 181:8-12.
24. Kornacki, J., A. West, and W. Firshein (1984) Proteins encoded by the trans-acting replication and maintenance regions of broad host-range plasmid RK2. Plasmid 11:48-57.

25. Schmidhauser, T., M. Filutowicz, and D.R. Helinski (1983) Replication of derivatives of the broad host-range plasmid RK2 in two distantly related bacteria. Plasmid 9:325-330.
26. Figurski, D.H., R.F. Pohlman, D.H. Bechhofer, A.S. Prince, and C.A. Kelton (1982) Broad host range plasmid RK2 encodes multiple Kil genes potentially lethal to Escherichia coli. Proc. Natl. Acad. Sci., USA 79:1935-1939.
27. Thomas, C.M., A.A.K. Hussain, and C.A. Smith (1982) Maintenance of broad host-range plasmid RK2 replicons in Pseudomonas aeruginosa. Nature 298:674-676.
28. Oka, A., N. Nomura, M. Morita, H. Sugisaki, K. Sugimoto, and M. Takanami (1979) Nucleotide sequence of small ColE1 derivatives: Structure of the regions essential for autonomous replication and colicin E1 immunity. Mol. Gen. Genet. 172:151-159.
29. Andres, I., P.M. Slocombe, F. Cabello, J.K. Timmis, R. Lurz, H.J. Burkardt, and K.N. Timmis (1979) Plasmid replication functions II. Cloning analysis of the RepA replication region of antibiotic resistance plasmid R6-5. Mol. Gen. Genet. 168:1-25.
30. Manis, J.J., and B.C. Kline (1977) Restriction endonuclease mapping and mutagenesis of the F sex factor replication region. Mol. Gen. Genet. 152:175-182.
31. Kolter, R., and D.R. Helinski (1978) Construction of plasmid R6K derivatives in vitro: Characterization of the R6K replication region. Plasmid 1:571-580.
32. Diaz, R., and W.L. Staudenbauer (1982) Replication of the broad host-range plasmid RSF1010 in cell-free extracts of Escherichia coli and Pseudomonas aeruginosa. Nucl. Acids Res. 10:4687-4702.
33. DeGraaff, J., J.H. Crosa, F. Heffron, and S. Falkow (1978) Replication of the nonconjugative plasmid RSF1010 in Escherichia coli K-12. J. Bact. 134:1117-1122.
34. Germino, J., and D. Bastia (1983) The replication initiator protein of plasmid R6K tagged with β-galactosidase shows sequence-specific DNA binding. Cell 32:131-140.
35. Vocke, C., and D. Bastia (1983) DNA-protein interaction at the origin of DNA replication of the plasmid pSC101. Cell 35:495-502.
36. Arai, K., and A. Kornberg (1981) Unique primed start of phage phiX 174 DNA replication and mobility of the primosome in a direction opposite chain synthesis. Proc. Natl. Acad. Sci., USA 78:69-73.

CONTROL OF PLASMID REPLICATION: THEORETICAL CONSIDERATIONS AND PRACTICAL SOLUTIONS

Kurt Nordström

Department of Microbiology
Biomedical Center
Uppsala University
Box 581
S-751 23 Uppsala, Sweden

INTRODUCTION

The life cycle of bacterial plasmids involves 2 different processes, replication (rep) and partitioning (par). Both these processes have to be controlled in order to secure stable inheritance of a plasmid in a growing population of bacteria. The crucial genetic information for (control of) replication (21) and partitioning (31,41) is present on the plasmids themselves. The 2 processes seem to be independent of each other since deletion of the par region of a plasmid does not affect the copy-number control although it results in unstable inheritance of the plasmid (41). Furthermore, the par region of 1 plasmid can be replaced by that of another, giving totally stable inheritance and the same copy number as that of the initial replicon (42). A third system adds to the stability of inheritance of plasmids, the ccd (control of cell division) system that inhibits cell division in cells containing only 1 copy of the plasmid (45).

Different plasmids are present in different copy numbers. I will discuss the mechanisms that control plasmid replication.

GENERAL PROPERTIES OF PLASMID REPLICATION

All plasmids seem to have a series of replication properties in common. They utilize host systems for their replication. Hence, the plasmid-borne genetic information needed for replication is

fairly small, 1-3 kb (kilobase pairs). This genetic information is often denoted the basic replicon (20). A basic replicon consists of 1 (or sometimes 2-3) origin(s) for replication, and, in some cases, but not always, the structural gene(s) (rep) for 1-3 proteins needed for replication (see also Tab. 2). These proteins are presumably needed for initiation of replication (although the biochemical function has not yet been clarified for any of the rep proteins); initiation is the rate-limiting step in replication and, at least for some plasmids, replication is controlled by regulation of the expression of a rep protein (24; Novick et al., unpub. data).

The population biology as well as the replication control systems (cop for copy-number control) of all or most plasmids have a series of properties in common (Tab. 1):

1.) Plasmid replication is autonomous and not linked to any specific event in the chromosome. replication cycle or in the cell cycle of the host. This conclusion is mainly based on the fact that the ratio between the number of plasmid copies and the number of chromosomal origins and termini varies with the growth rate (7,11).

2.) There is a widespread in inter-replication times in the plasmid population, i.e., the daughter of a plasmid molecule can replicate much earlier or much later than one generation after its formation. This conclusion is based on Meselson-Stahl density shift experiments (2,12,53). (This is often referred to as random selection.) The spread in inter-replication times has got a minimum value because plasmids under replication are not available for a

Tab. 1. General characteristics of plasmid replication.

Characteristics of plasmid replication	References
1. Autonomous, i.e. independent of chromosome replication and cell division cycles.	7,11
2. Wide spread in interreplication times	2,12,38,53
3. Replication spread over the entire cell cycle	1,12
4. Defined copy numbers defined by plasmid, host, and growth conditions	7,9,11,28,34, 40,66
5. Deviations from normal copy number adjusted.	
6. Copy mutants exist. Some are dominant others recessive to the wild type	3,34,40,54,66, 74
7. Cloned DNA may cause inhibition of replication, incompatibility (inc or cop)	65

second replication event (38); this eclipes period can be fairly long, e.g., more than 25-30 min for oriC plasmids (23).

The spread in inter-replication times causes randomization during replication which together with random assortment for partitioning at cell division forms the basis of plasmid incompatibility (Fig. 1) (44).

3.) Plasmid replication is spread over the whole cell cycle. This conclusion is based upon 2 kinds of experiments, Meselson-Stahl density shift experiments and analysis of plasmid replication in cells of different size (1,12).

4.) In exponentially growing populations of plasmid-carrying bacteria, plasmids are present in distinct copy numbers. These are defined by the plasmid (34,40,66), the host (9,28), and the growth conditions (7,11). It should be stressed that in the steady state each plasmid copy replicates, on the average, once during a doubling time; this is an average value, and does not apply to the individual plasmid copy (see point 2 above).

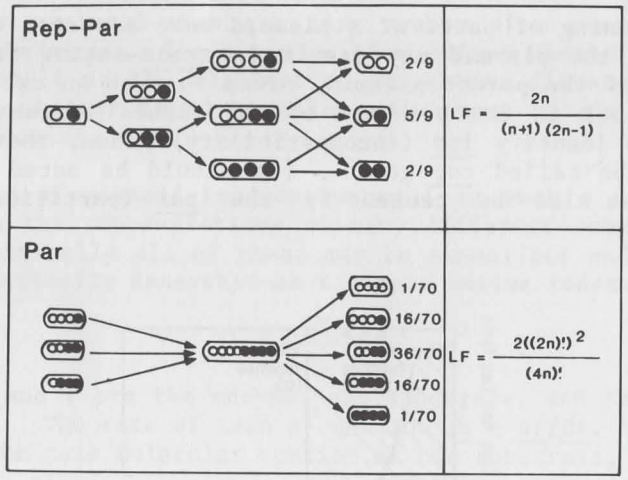


Fig. 1. Plasmid incompatibility as a consequence of randomization during replication and partitioning. The open and full circles represent 2 differently marked plasmids: In the Rep-Par case, the 2 plasmid species have the same replication and partitioning systems; in the Par case, 2 different replicons have the same Par system. The ratios to the right give the frequency of the different combinations of 2 plasmid species after cell division. LF represents the rate of reduction of the relative size of heteroplasmid population during each cell cycle.

5.) The same defined copy number is obtained after introduction (by transfection, transduction, or conjugation) of 1 plasmid copy into a plasmid-free cell. Similarly, perturbations giving average copy numbers higher or lower than the defined one are gradually corrected during growth eventually resulting in the defined average copy number. Hence, the number of replications per plasmid and cell cycle (which is in average exactly 1 in the steady state) is more than 1 at copy numbers lower than the defined one and less than 1 at copy numbers higher than the defined one (Fig. 2), i.e., the rate of replication per plasmid copy falls with increasing plasmid concentration (the hatched areas in Fig. 2).

6.) Mutations in the plasmid (34,40,66) and (in some cases) the host chromosome (9,28) may result in an increased plasmid copy number. Such plasmid mutants are called copy mutants (Cop, cop). Some of these are dominant, others recessive to the parent plasmid. This means that there is a preferential loss of the wild type from cells also carrying a dominant copy mutant, whereas the copy number of a recessive copy mutant is reduced by the simultaneous presence of the wild-type parent plasmid (3,74). There are also copy mutants with a lower copy number than that of the wild-type. Some copy mutants have become compatible with their wild-type parent (54,74).

7.) Cloning of parts of a plasmid onto a vector that is compatible with the plasmid may result in trans-acting inhibition of replication of the parent plasmid, often to such an extent that the plasmid is lost in the presence of the cloned fragment; this has been used to identify inc (incompatibility) genes, that more suitably should be called cop genes. [It should be noted that incompatibility can also be caused by the par (partitioning system)

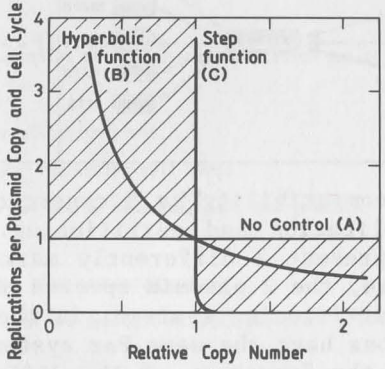


Fig. 2. Schematic presentation of the kinetics of control of replication. The control curves have to lie within the hatched areas. Curves B and C show 2 examples of control kinetics.

(42).] In some cases a cop^+ gene may reduce the copy number of a cop mutant without expressing incompatibility, i.e., causing loss of the mutant plasmid (5,33).

THEORETICAL MODELS FOR PLASMID REPLICATION AND ITS CONTROL

I will now take up a few simple theoretical systems for control of replication and discuss how they meet the conditions of Tab. 1. In doing this, I will treat the plasmid population in a culture as if it formed 1 pool; the cell is irrelevant for such a discussion.

Passive Control of Plasmid Replication

When bacteria are growing exponentially in a medium that contains all nutrients in saturating amounts, steady states are established that are characterized by constant ratios between all components of the cell, i.e., the concentration of each cell component does not change with time. The actual concentration of the various cell constituents at steady state growth is in some cases due to active control, but presumably the concentration of many cell components is merely a consequence of the rate constants of the numerous biochemical reactions that take place in a cell. Consequently, it cannot be excluded that the copy number of a plasmid could be passively maintained. The question then arises whether such a system might result in the replication properties discussed above and particularly whether it might allow adjustment of the copy number after perturbation.

The rate of plasmid replication will in such a system be dependent upon the concentrations of many different compounds. However, mathematically all of these may be summarized and replication may be schematically described as a simple enzyme reaction:

$$S \underset{}{\overset{E}{\rightleftharpoons}} P \qquad (1.)$$

where E, S, and P are the enzyme, the substrate, and the product of the reaction. The rate of such a reaction ($v = dP/dt$, but since the product is the same molecular species as the substrate, $v = dS/dt$):

$$v = V \cdot S/(K + S) \qquad (2.)$$

V is the maximal rate of this reaction, S is the concentration of the substrate, K is the Michaelis constant.

At saturation, i.e., $S \gg K$ the rate becomes constant, independent of S. The rate of replication of each plasmid copy will drop as S increases:

$$v/S = V/(K + S) \xrightarrow[S \gg K]{} V/S \qquad (3.)$$

i.e., at higher S values, the rate function is an hyperbolic function (as is the rate curve for at least some plasmids, see below).

Let us briefly discuss whether such a system might fit conditions 1-7 in the preceding section and Tab. 1.

Replication of the plasmid would, of course, be a purely statistical event. Hence, conditions 1-3 would clearly be fulfilled. The system would also lead to stable copy numbers (n values). At steady state, the rate can be expressed as the formation of n molecules in a volume (a cell) that, at the start, has n molecules. Hence,

$$v = dS/dt = n \cdot \ln 2/T = N \cdot \ln 2 \cdot n/T(K' + n) \qquad (4.)$$

where T is the generation time and N is the maximum rate expressed as number of replications per cell and cell cycle. K' is the Michaelis constant expressed as number of plasmid molecules per baby cell. This gives the steady state copy number:

$$n = N - K' = \text{constant} \qquad (5.)$$

Depending on the rate constants, the n value can be at any part of a Michaelis-Menten curve (Fig. 3).

Since the rate of the reaction per plasmid copy decreases by increasing concentration of the substrate (Fig. 3), the system will also be able to adjust aberrations in copy number (Fig. 4). This

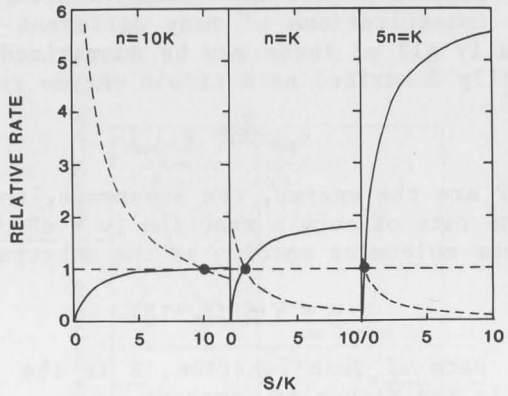


Fig. 3. Passive control of plasmid replication. The graph shows 3 examples of control kinetics (eq. 5.). The solid lines represent the rate of replication (eq. 2.); the dotted lines, the rate of replication per plasmid copy (eq. 3.); and the solid circles, the steady state values.

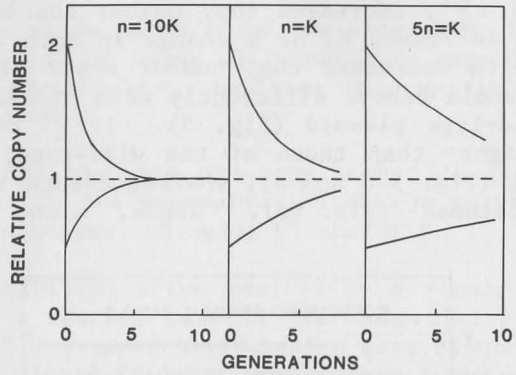


Fig. 4. Passive control of plasmid replication: kinetics of restoration of steady states after perturbations of the copy number. Conditions are as in Fig. 3. The dotted lines represent the steady state conditions.

adjustment will be fairly rapid, but the time required to regain the steady state will be different at different values of the enzyme constants (Fig. 4).

Plasmid replication is specific and the specificity has to reside in the nucleotide sequence of the plasmid. If 2 plasmids carry the same specificity region, they will compete for the reaction and, hence, they will show incompatibility.

The wide spread in inter-replication times causes variations in the ratio between the 2 plasmids, deviations that cannot be corrected (see Fig. 1).

Mutations in the specificity region could change V as well as K, i.e., result in change in copy number (copy mutants). The simultaneous presence of a copy mutant plasmid and the wild-type parent plasmid in a cell would resemble an enzyme reaction with 2 substrates present. In such a case, the substrate will be competitive inhibitors of each other:

$$E + S_1 \rightleftharpoons C_1 \longrightarrow P_1 + E \qquad (6.)$$

$$E + S_2 \rightleftharpoons C_2 \longrightarrow P_2 + E \qquad (7.)$$

which gives:

$$v_1 = N_1 \cdot n_1/(K_1 + n_1 + n_2 \cdot K_1/K_2) \qquad (8.)$$

$$v_2 = N_2 \cdot n_2/(K_2 + n_2 + n_1 \cdot K_2/K_1) \qquad (9.)$$

Since n = N - K', increased copy number can be obtained by a decreased K', an increased N, or a change in both (Fig. 5). Similarly, mutants with decreased copy number would also be possible. The cop mutants would behave differently when present in cells also carrying the wild-type plasmid (Fig. 5). If K' of the mutant is lower or N is higher than those of the wild-type, the cop mutant would be dominant (Fig. 5 a and b), whereas change in both K' and N may give recessiveness (Fig. 5c). Hence, also condition 6 is

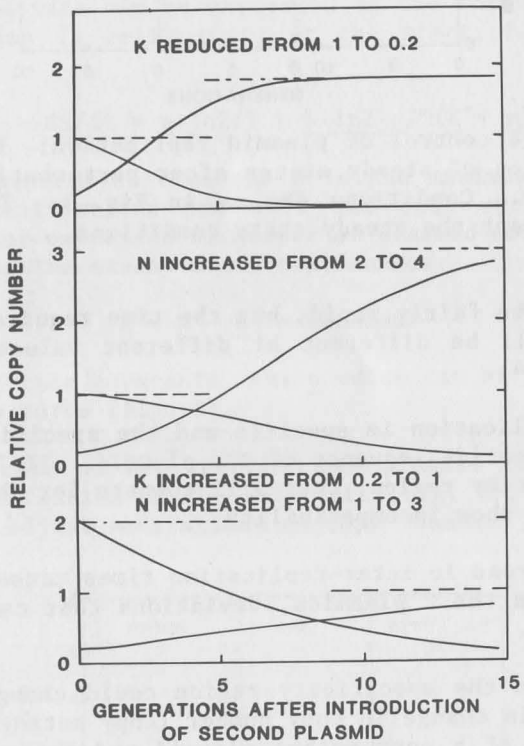


Fig. 5. Passive control of plasmid replication and incompatibility: kinetics of replication after creation of a heteroplasmid situation. a (top) and b (middle): the wild-type plasmid has been given the K' and N values of 1 and 2, respectively, whereas the mutant in the top case has a 5-fold lower K' value, and, in the middle case, a 2-fold increased N value. The mutant was introduced at a 5-fold lower concentration than the steady state concentration of the wild-type plasmid. c (bottom): the wild-type has a K' value of 0.2 and an N value of 1, whereas the mutant has the K' and N values 1 and 3, respectively. The wild-type plasmid was introduced at a 10-fold lower concentration than the steady-state concentration of the mutant.

filled. However, there seems to be one exception; it is hard to explain how mutation might cause an increase in copy number and also a total change of specificity, resulting in compatibility between the mutant and its parent.

Host mutations changing the copy number of a plasmid would be caused by changes in the concentration of the enzyme (E) or in the properties of the enzyme, changing K' and/or N.

The incompatibility often exerted by a cloned fragment from a plasmid will be a special case of the competitive inhibition discussed above. If the specificity region were cloned onto a compatible plasmid, the cloned fragment would cause a reduction in the replication frequency of its parent plasmid, i.e., the cloned fragment would express negative control of replication and express incompatibility. Hence, this passive control is an example of negative control.

In conclusion, the simple passive control would give replication properties as 1-7 discussed above. Furthermore, the discussion shows that negative control does not require the existence of specific inhibitors.

However, there is no real indication of the existence of saturation kinetics as a device for control of plasmid replication. It is very hard to imagine what the rate limitation could be, since the system has to be plasmid-specific; there are at least 20 compatibility groups in Escherichia coli. Compatible plasmids should use different rate-limiting reactions although, biochemically, replication of all plasmids is very similar.

Furthermore, there is a quantitative aspect: the wet weight of an E. coli cell is above 10^{-12} g corresponding to a volume of 10^{-15} l. Hence, 1 plasmid molecule per cell represents a plasmid concentration of 2×10^{-9} M. This is a very low concentration. During one generation (20 min or more) a low-copy-number plasmid (n = 2-4) replicates only very few copies per cell; hence, the turnover number is extremely small, <<1 per enzyme molecule and min. This argues for an active negative control rather than for a passive control. A hard argument is, of course, that in those cases where anything specific is known, the plasmid codes for a negative control element (see below). However, this does not exclude that there, somewhere, might be a plasmid that uses a very simple passive control of its replication. This might, for example, apply to some cop mutants where the copy number is very high which might result in host factors/systems being rate-limiting.

Negative Control of Plasmid Replication

During the vegetative cycle, phages replicate as fast as possible. Phage λ, for example, goes through an early phase of expo-

nential replication during which the phage DNA population doubles every 2-3 min (4,77). Plasmids, on the other hand, replicate on average exactly once per cell cycle, i.e., much more rarely than vegetative phages do. Hence, plasmids are most of the time nonreplicating. Consequently, plasmid replication is inhibited most of the time. This requires some sort of negative control whose function is to titrate the concentration of the plasmid. The rate of replication per plasmid copy decreases with the concentration of the plasmid. This might be achieved by passive control (see the previous section). The alternative would be a system with a negative loop. Negative control was first proposed by Pritchard (46) and the so-called Inhibitor-Dilution-Model has been discussed in several reviews (47,48). Therefore, I will not discuss these models, but try to generalize the discussion even further. There seem to be 2 types of models proposed: one where the inhibitor is formed constitutively (47,48), the other where it is autoregulated (58). Some general properties of these models are discussed in the following sections.

Negative control by a constitutively formed repressor. The simplest system of this type would be one in which the constitutively formed repressor acts at the origin of replication (or at the site where the first event in replication occurs).

$$\mathrm{Cop} + \mathrm{ori} \rightleftharpoons \mathrm{Cop\text{-}ori} \qquad (10.)$$

$$\underline{dS}/\underline{dt} = k_1 \cdot \underline{ori} \qquad (11.)$$

$$\underline{ori} + \underline{Cop\text{-}ori} = \underline{S} \qquad (12.)$$

$$\underline{Cop} \cdot \underline{ori}/\underline{Cop\text{-}ori} = \underline{K}. \qquad (13.)$$

In the steady state, $\underline{Cop} = k_2 \cdot \underline{S}$ (14.)

Hence, $\underline{ori} = \underline{K} \cdot \underline{S}/(\underline{K} + k_2 \cdot \underline{S})$ (15.)

and $\underline{dS}/\underline{dt} = k_1 \cdot \underline{K} \cdot \underline{S}/(\underline{K} + k_2 \cdot \underline{S}) = S \cdot \ln 2/T,$ (16.)

which gives $S = K(k_1 \cdot T/\ln 2 - 1)/k_2.$ (17.)

Hence, conditions 1-4 are clearly met. Furthermore, the discussion of conditions 5-7 will be very much the same as for autoregulation (see next section).

Autorepressor control. Sompayrac and Maaløe (58) suggested an autorepressor model for control of replication of the E. coli chromosome. Murotsu and Matsubara (36) concluded that λ dv is an example of a plasmid that uses an analogous system for control of its replication (see below).

An autorepressor can either also be the positively needed Rep function or a separate one; in the latter case, the repressor and the Rep function form an operon; see plasmid λdv in Fig. 6. The rate of replication is determined by the concentration of the Rep function. The rate of formation of Rep is determined by the concentration of operator-promoter (op) that has not bound the inhibitor Cop. The concentrations of Cop, op, and Rep are denoted Cop, op, and Rep, respectively:

$$\mathrm{op} + \mathrm{Cop} \rightleftharpoons \mathrm{op\text{-}Cop}, \qquad (18.)$$

which gives $\underline{op} \cdot \underline{Cop}/\underline{op\text{-}Cop} = \underline{K},$ (19.)

and $\underline{op} + \underline{op\text{-}Cop} = \underline{S},$ (20.)

where S is the substrate of the reaction, the replicon. This gives

$$\underline{op} = \underline{K} \cdot \underline{S}/(\underline{K} + \underline{Cop}) \qquad (21.)$$

In the steady state, the concentrations of all components in the cells are constant. This also means that the rate of formation of any component is proportional to that of any other. Hence,

$$\underline{Rep} = k_2 \cdot \underline{S} \text{ and } \underline{dRep}/\underline{dt} = k_2 \cdot \underline{dS}/\underline{dt} \qquad (22.)$$

and $\underline{Cop} = k_3 \cdot \underline{S}$ and $\underline{dCop}/\underline{dt} = k_3 \cdot \underline{dS}/\underline{dt}.$ (23.)

Since $\underline{dRep}/\underline{dt} = k_4 \cdot \underline{op},$ (24.)

$$(\underline{dS}/\underline{dt})k_2 = \underline{dRep}/\underline{dt} = k_4 \cdot \underline{op} \qquad (25.)$$

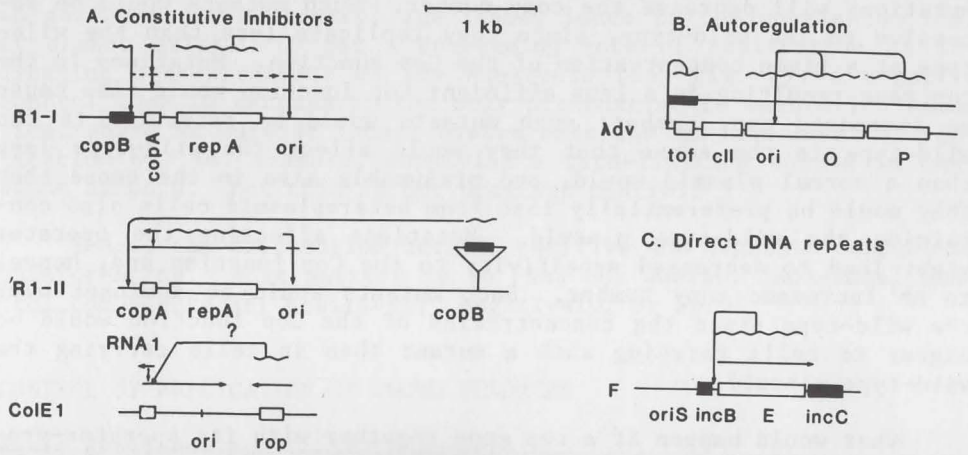


Fig. 6. Control circuits of the basic replicons of a few plasmids.

and $$dS/dt = k_4 \cdot op/k_2 = k_4 \cdot K \cdot S/k_2(K + k_3 \cdot S). \quad (26.)$$

The steady state so obtained will give a defined n value, which can be calculated as follows: The population of plasmids grows exponentially with a defined generation time, T.

$$dS/dt = k_1 \cdot S = S \cdot \ln 2/T. \quad (27.)$$

Hence, $$n \cdot \ln 2/T = k_4 \cdot K \cdot n/k_2 \cdot (K + k_3 \cdot n) \quad (28.)$$

and $$n = K(k_4 \cdot T/\ln 2 - k_2)/k_2 \cdot k_3. \quad (29.)$$

The autorepressor system is of course truly statistical and conditions 1-3 in Tab. 1 are met, and so is condition 4, as was demonstrated in equation (29.).

What about condition 5, the ability to compensate for deviations from the steady state level? If a cell happens to get more copies of the replicon, e.g., by uneven distribution at cell division, the rate of replication in the cell will increase. However, the output of Cop will also increase and gradually slow down transcription from op, and decreasing the probability of replication to <1 per plasmid copy and cell division. The opposite will be true for cells that happen to get fewer copies than the average cell. The actual kinetics will be dependent upon a series of conditions, e.g., the stability of Rep and Cop.

Copy mutants may be obtained by mutations that change any of the constants k_2, k_3, k_4, and K; the mutations might be in the op region or the cop or rep genes or in the target for the Rep protein. Mutations in the rep gene or the Rep target as well as promoter-down mutations will decrease the copy number. Such mutants would be recessive to the wild-type, since they replicate less than the wild-type at a given concentration of the Cop function. Mutations in the cop gene resulting in a less efficient Cop function would also cause an increased copy number; such mutants would be recessive to the wild-type in the sense that they would affect the wild-type less than a normal plasmid would, and presumably also in the sense that they would be preferentially lost from heteroplasmid cells also containing the wild-type plasmid. Mutations affecting the operator might lead to decreased sensitivity to the Cop function and, hence, to an increased copy number. Such mutants would be dominant over the wild-type since the concentration of the Cop function would be higher in cells carrying such a mutant than in cells carrying the wild-type plasmid.

What would happen if a cop gene together with its operator-promoter were cloned onto a cloning vehicle? At high ratios between the copy number of the vector and the plasmid the concentration of op + op-Cop would remain constant and equal to the concentration of the vehicle; T; at lower ratios, the plasmid will also contribute to op + op-Cop

$$op + op\text{-}Cop = T \quad (30.)$$

$$dCop/dt = k_5 \cdot op \quad (31.)$$

Equation (19.) then gives

$$op = K \cdot T/(K + Cop) \quad (32.)$$

$$dCop/dt = k_5 \cdot op = k_5 \cdot K \cdot T/(K + Cop). \quad (33.)$$

However, in the steady state

$$dCop/dt = \ln 2 \cdot Cop/T = k_5 \cdot op, \quad (34.)$$

hence $$K \cdot T/(K + Cop) = \ln 2 \cdot Cop/k_5 \cdot T \quad (35.)$$

and $$Cop = -K/2 \pm \sqrt{K^2/4 + K \cdot k_5 \cdot T \cdot T/\ln 2}. \quad (36.)$$

Hence, a cloned cop gene will express inhibition of replication (Inc) at an extent that increases with the concentration of the gene.

Positive Control of Plasmid Replication

The membrane-site model of Jacob, Brenner, and Cuzin (18) has been used, not completely correctly, as the prime model of positive control. A lot of effort has been used to distinguish between positive and negative control of replication. However, all this has been a waste of time and effort, since nobody seems ever to have tried to define what positive control of replication means. A try to define positive control inevitably leads to the conclusion that it cannot exist. To get a decreasing rate of replication at increasing concentrations of the substrate (see Fig. 2), a negative control loop is necessary; the only alternative is saturation kinetics, where an outside (host) system sets the maximum level of replication by, for example, supplying a rate-limiting enzyme in constant amounts. But this is not positive control.

Hence, the distinction between positive and negative control is uninteresting. What matters is to find out whether individual control components act positively, negatively, or both.

CONTROL OF REPLICATION OF KNOWN PLASMIDS

Basic Replicons and Their Components

Basic replicons. Natural plasmids can be any size from a few

kb to hundreds of kb. However, large plasmids carry a whole series of genes that are concerned with functions other than plasmid maintenance. The size of a replicon can be reduced by using restriction endonucleases in vitro (e.g., 65). Kollek et al. (20) introduced the concept basic replicon for the smallest piece of plasmid DNA that is able to replicate. I prefer a more limited definition, namely a basic replicon is the smallest piece of DNA that replicates with the wild-type copy number.

Basic replicons normally form a continuous piece of DNA the size of which in most cases is 1.0-2.5 kb (Tab. 2). Hence, a basic replicon is fairly small, with a potential of coding for 3-4 proteins, because plasmids use host functions for their replication. As a comparison, conjugal transfer requires one order of magnitude more DNA (76).

Some large plasmids have more than one basic replicon; presumably one of these is solely or preferentially used.

Basic replicons consist of origins, cop genes, and (in some cases) rep genes (Tab. 2). Fairly little is known about how replication is initiated; plasmid ColE1 is an exception here (15).

Origins of replication. Replication does not start at random but at defined origins (15). Some plasmids have more than 1 origin. Normally, only 1 of the origins seems to be active; however, a word

Tab. 2. Functions coded for by some plasmid basic replicons.

Plasmid	Size of basic replicon (kb)	Origin	Cop function	rep genes
ColEI	1-1.5	1	RNAI Rop Protein	None
R1(FII)	2.5	1	CopA-RNA CopB Protein	repA
pT181		1	RNA	repC
λ dv	2	1	Cro protein	O,P
F	1.5	oriV,oriS		repE
R6K		α,β,γ	Direct repeats	pir
PI		1	of DNA + ?	
RK2		1		
RSF1010	5kb	1	?	repA,B,C

of caution is needed since very little direct experimentation has been devoted to investigate this. Plasmid R6K is an example of a plasmid in which 3 origins (α, β, and γ) are all used with about equal frequency (22).

Origins are not well-defined. In most cases they are identified by electron microscopy as bubbles on early replicative intermediates (ss Ref. 15,65). Plasmid ColE1 uses an RNA primer and the exact location of the shift from RNA to DNA synthesis has been identified to be any of 3 consecutive base pairs (68). Similarly, plasmid oriC starts its replication at 3-4 closely linked base pairs (14).

Origins were once concluded to have a high degree of secondary structure with the possibility of forming stem-loop structures. However, the so identified origin of FII plasmids (51) was recently found to be outside of the functional origin [identified by in vitro replication (29)]. However, it is possible that the region of secondary structure might have a function in determining the activity of the origin.

Rep functions. Some plasmids (e.g., ColE1) do not require any plasmid-coded functions for their replication. However, it is fairly common that plasmids code for 1-3 Rep functions (Tab. 2). These are supposed to be involved in initiation of replication, although the exact function has not been identified for the Rep function of any plasmid. Since there are in vitro replication systems for plasmids R6K (16,17), R1 (10,29), and pT181 (19), progress can be be expected in the biochemical understanding of initiation of replication.

The formation of Rep protein is rate-limiting for replication of at least some plasmids [FII (24), and F (57)]. There are at least 2 cases where a Rep protein is mainly cis-acting [the λ O protein and the FII RepA protein (29)]; this means that they preferentially replicate the DNA molecule from which they were coded. They act in trans only if the cis-target is absent (29); the target is the origin (ori) of replication. The λ O protein is clearly very unstable (29) and the RepA protein seems to be "destroyed" by the presence of oriR1 or stoichiometrically used in replication (29).

De novo protein synthesis is needed for each round of replication of FII plasmids (10,75).

Cop functions. Deletion and cloning analysis of several plasmids have always led to the identification of DNA sequences that are acting in trans as inhibitors of replication. A careful analysis (genetic or, in a few instances, biochemical) have identified 3 types of inhibitors (Tab. 2), small proteins (5,30,33), small RNA molecules (3,35,62,69), and a series of direct repeats of DNA (22,60,61,64,67,73). In the last case, formation of RNA has not

been completely ruled out. However, it appears that DNA itself may cause trans-inhibition of replication.

The small RNAs have a high degree of secondary structure allowing the formation of long stems with small loops (3,35,62,69). The specificity rests on the loops (3,35,62) and the inhibitory effect is specified by interaction between RNA and RNA (25).

Control kinetics. The rate of replication per plasmid copy decreases with increasing plasmid concentration (hatched areas in Fig. 2). The actual kinetics is not known in more than 2 cases; plasmids F and R1 (a member of the FII group). The control curve of the latter plasmid was recently discussed in detail (43) and shown to be an hyperbolic one, whereas it is more like a step function for the former plasmid (72).

Brief Presentation of a Few Systems

The presentation of plasmid replication control systems is not intended to be a complete review, but aims at showing the principles used by some plasmid systems (see Fig. 6).

Plasmids using small RNA molecules as main copy number control device. Three different plasmids clearly use small (80-100 nucleotides) RNA molecules as their main control device, namely the E. coli plasmids ColE1 (35,69) and those of the FII group (3,54,62), and the Staphylococcus aureus plasmid pT181 (Novick, pers. comm.) (Tab. 2).

There are several similarities between these systems. The main control system is an RNA molecule with a high degree of secondary structure; the specificity resides in a small loop at the end of a long stem (3,35,62,69). The target for the small RNA is a long transcript formed from the complementary strand. Hence, the primary interaction is one between 2 complementary RNA species (26,35,62, and Novick et al., unpub. data). The specificity (and the specific activity) of the interaction can be drastically affected by single base-pair substitutions, some of which completely change the incompatibility properties of the system (35,62). The biochemistry of the RNA-RNA interaction is not known, but the formation of an RNA duplex is an appealing possibility. In all 3 cases, the effect of the interaction is down-stream on the long transcript, reducing the ability to translate a rep gene (FII and pT181) (26) or reducing the ability to process the long transcript into a primer (ColE1) (35,69). The Rep protein of the FII group is (mainly) cis-acting (29), whereas that of plasmid pT181 is acting both in cis and in trans (Novick et al., unpub. data).

These 3 plasmids may be regarded as examples of plasmids using a simple negative control by a constitutively formed repressor (see

above); the target is not the ori but an earlier step that is rate-limiting and that is post-transcriptional.

The kinetics of the replication control system are best known in the FII group. Plasmid R1 of this group forms an unstable RNA molecule (half life 1-2 min., Ref. 62). This means that the concentration of the copA-RNA always is proportional to the concentration of the plasmid and the target (43). The expression of the repA gene is proportional to the concentration of plasmid, but inversely proportional to the concentration of the inhibitor and, hence, the total rate of formation of the RepA protein in a given volume is constant. This was previously found experimentally in shifts between different copy numbers (11,13). Hence, the control curve is hyperbolic (Fig. 2).

Interestingly, another replication system, namely transposition of transposon Tn10 is negatively controlled by a small transcript that is complementary to the early region of the transposon message (56).

Since the RepA protein is cis-acting, a mutation increasing the output of RepA protein is a cis-acting mutation and the mutant plasmid is dominant over the wild-type. This is not the case for plasmid pT181 where the RepC protein is acting both in cis and in trans (Novick et al., unpub. data). Hence, the increased pool of RepC protein can be used by the mutant as well as the wild-type plasmid.

Plasmid ColE1 has got a second cop gene (rop; repressor of primer), coding of a small acidic protein (5). Deletion of the rop gene increases the copy number several-fold. Addition of extra rop genes does not decrease the copy number of a wild-type plasmid; hence the Rop protein does not exert incompatibility. The Rop protein seems to act together with the RNAI, maybe to terminate transcription (not at all clear at present) (5).

The secondary control system of FII plasmids, the CopB system. Plasmids of the FII group superficially resemble the ColE1 group (Fig. 6). They also contain the structural gene, copB, coding for a small protein (in this case basic, though) (33). Deletion of the copB gene increases the copy number several-fold; addition of extra copB copies does not change the copy number of the wild-type plasmid. However, mutants deleted of the copB gene are very sensitive to extra copB copies; hence, copB expresses incompatibility towards the copB mutants but not towards the wild-type (33,49). However, there is a difference between the FII and the ColE1 group. The target for the CopB protein is a promoter and the effect of the protein is to almost totally switch-off a second promoter (repAp) (25). Womble and Rownd (unpub. data) and Riise (unpub. data) have demonstrated that this promoter is derepressed at concentrations of the CopB protein below the wild-type level. Similarly, Riise et al. (49)

showed that insertion of the CopB gene and its promoter into an R1 replicon deleted of the CopB gene and its promoter gave a replicon with a functional copB gene at a new location (Fig. 6). This plasmid has a copy number 1/4-1/3 of that of the wild-type replicon. The control kinetics of this replicon has not been worked out (and is hard to study, because of the very low copy number) but a fair guess is that the control curve would be steeper than a hyperbolic one.

Finally, it should be stressed that a plasmid in which the active copB gene has been moved to a new location still is a plasmid of the same type as the parent plasmid, but the plasmid now has 2 independent constitutively formed repressors (Fig. 6).

Plasmid λdv using a simple autorepressor control system. Plasmid λdv controls its replication by an autorepressor system. The autorepressor is the Cro protein, which controls a transcript that, downstream of the cro message, contains 2 rep genes (O and P) (30). The O protein is cis-acting and the target is a region with 4 direct repeats in the O gene, which also contains the replication origin (71). The O protein is unstable and the replication rate is either determined by the output of the O protein, by transcriptional activation of ori, or both (15,70).

Murotsu and Matsubara (36) have shown that plasmid λdv fits well into the Sompayrac-Maaløe (58) autorepressor theory. They have isolated promoter and operator mutations and these behave in the predicted way with respect to copy number, incompatibility, and expression of the control gene (cro).

Lee and Bailey [Plasmid 11 (1984) 151-165 and 166-177] have presented a mathematical model for replication of plasmid λdv and its copy mutants. The model is based upon the wealth of quantitative information that is available for phage λ. The authors have been able able to predict the correct copy number as well as several other replication parameters of the plasmid. They also predict that the control curve is very close to an hyperbolic curve (see Fig. 2).

Plasmids with direct repeats as cop/inc genes. Several plasmids have cop-DNA that expresses its function without any sign of formation of gene products. The cop-DNA has got a series of carefully spaced direct repeats (18-22 bp). This applies, for example, to plasmids F (67,73), R6K (22,60), P1 (Chattoray and Yarmolinsky, pers. comm.), and RP1 (RK2) (61,64). On top of this they (at least some of them) also seem to have an autorepressor system where a rep gene is autoregulated (22,57,59). A lot is known about plasmid R6K, where the rep gene (pir) is autoregulated (22,59). Mutations in the pir gene may cause an increase in copy number (59) but there are also data that contradict the conclusion that the pir gene product

(the π protein) is rate-limiting (22). The function of the repeats is obscure and very little is known about the control parameters.

Plasmid F is better known with respect to control kinetics. Tsutsui and Matsubara (72) showed that F replication is totally switched-off at plasmid concentrations twice that of the wild-type level. Hence, the control curve seems to be a step function. Expression of the E gene is autoregulated (57). Tolun and Helinski (67) have shown that a 55-bp fragment containing 2 of the 9 direct repeats of the basic F replicon expresses a weak inhibition of replication. Tsutsui et al. (72) have shown that the number of repeats in a cell controls replication: the location or orientation does not seem to matter.

What is the molecular basis for the trans-acting negative control of replication of plasmid F? There is no indication of the formation of any gene product from this region. The suggestion has then been that the direct repeats in the DNA nucleotide sequence titrates a positive factor [Tsutsui et al. (73)]. Some workers in the field seem to be hesitant to this as if DNA were of a different stuff than all other macromolecules. However, I can't see any difference between the binding of an inhibitory protein to a DNA sequence and the binding of a positively needed protein (Rep) to an inhibitory DNA segment (rpt for repeat).

If we, for simplicity, assume that Rep_o is constant, then:

$$Rep + rpt \rightleftharpoons C \qquad (37.)$$

$$Rep \cdot rpt/K = C \qquad (38.)$$

$$rpt = a \cdot S \qquad (39.)$$

$$C + Rep = Rep_o. \qquad (40.)$$

This gives

$$Rep = K \cdot Rep_o/(K + a \cdot S). \qquad (41.)$$

Hence, the frequency of replication ($dS/dt = k_1 \cdot Rep$) drops with increasing substrate concentration (S). However, the decrease is slow (at high S values dS/dt is inversely proportional to S). The equations are based on a high ratio rpt/Rep_o. If this ratio is closer to 1, a second condition has to be added.

$$rpt + C = rpt_o \qquad (42.)$$

If K is small, the situation will be similar to titration and almost every molecule of Rep will bind to rpt as long as these are in surplus. If there is a slow but steady production of Rep, suddenly all Rpt will be bound by Rep and free Rep molecules become available. Replication then supplies new Rpt that bind all free Rep molecules.

Hence, there will be a switch-on/switch-off of replication (a step function). However, this requires either that the number of Rep molecules is very small (maybe 20 per cell for plasmid F; some proteins are present in such low concentrations) but still synthesized steadily or that each repeat binds many Rep molecules (either as a cluster or by modifying them by binding and hence catalytically destroying Rep activity).

Nothing is known about what positive factor binds to the repeats, although the 29 k E protein of F is a prime suspect. Nothing seems to be known about the amount of 29 k protein formed. An intriguing fact is also that this and analogous proteins in other plasmid systems are autoregulated (22,57,59), which seems to be hard to understand; autoregulation of a titrated Rep function seems to be a contradiction. One possibility could be that the protein is bifunctional (as suggested by Molin et al., unpub. data) with one part being inhibitory and the other being a Rep function. This could keep autoregulation intact and allow destruction of the Rep function. Possibilities of establishing the identity of the Rep function and the protein that binds to the repeats are now clearly at hand for several of the plasmids that have repeats in their DNA nucleotide sequence as a main part of their replication control system.

CONTROL OF CHROMOSOME REPLICATION

Does chromosome replication obey the rules in Tab. 1? The answer is partly no. 1) Cooper and Helmsetter (8) have clearly shown that initiation of chromosome replication is not linked to a specific part of the cell division cycle. 2) At rapid growth, 2 or more initiations of chromosome replication take place per cell and cell cycle. However, the spread in inter-replication times is much less for the bacterial chromosome than for plasmids (8,12). 3) Similarly, multiple initiations essentially coincide in time and do not spread over the whole cell cycle (8). Requirements 4) and 5) are fulfilled. 6) Mutations exist that affect the copy number of the chromosome (52,63). However, these affect RNA polymerase and not specific *cop* genes. Finally, 7) has not been tested experimentally.

How do we explain that multiple initiations at chromosomal origins spread very little in time? One possibility would be that the control curve is a step function and that the *cop* gene is located some distance from the chromosomal origin. Multiple initiations at the same origin may be made impossible by steric reasons. Presumably, the newly replicated DNA is not in a form that is suitable for replication immediately after replication. This would resemble the slow replication cycle (from CCC-DNA to CCC-daughter-DNA) of (some) plasmids including *oriC* plasmids (23,75).

There is no direct evidence for the existence of a negative control element involved in chromosome replication. So far, work with *oriC* plasmids has not contributed much to the understanding of replication control (see brief discussion in Ref. 39).

Since there seem to be some differences between chromosome and plasmid replication, it might be a fruitful approach to turn the matter upside down. Can plasmids run the chromosome and, in such a case, what are the characteristics of the chromosome replication and the cell-division cycles? The answer to the first question is yes. Nishimura et al. (37) showed that plasmid F can integratively suppress a *dnaA(ts)* mutation. Chandler et al. (6) found that also plasmid R100 is able to perform integrative suppression; they also reported that the R100 origin is used at temperatures nonpermissive for chromosomal replication. Molin and Nordström (32) reported that an integrated plasmid R1 expresses CopA activity and is sensitive to CopA. This should make it possible to study the chromosome replication and cell-division cycles. However, these strains grow poorly in minimal medium at high temperature, perhaps due to effects on the membranes because *dnaA* expression is autoregulated. However, preliminary data in our laboratory (Luud Koppes) indicate that the size distribution of *intR1* cells at 42°C is much broader than that of normal *E. coli* strains. We are presently engaged in the construction of *dnaA*$^+$ strains where *oriC* has been replaced by the R1 and F basic replicons.

FINAL COMMENTS

Studies of control of plasmid replication have now reached a state where the mechanisms involved gradually are being unraveled. Clearly, negative control is involved, but the inhibitors can be DNA, RNA, or protein. Control is exerted by regulating transcription and/or translation rather than by interfering with the origin of replication and the biochemical events that take place there. In some cases, in vitro and in vivo studies of the quantitative aspects of replication control are possible. This will require careful measurements of binding constants, rate constants, etc.

ACKNOWLEDGMENTS

This work was supported by the Swedish Natural Science Research Council and the Swedish Cancer Society. I am grateful for numerous discussions throughout the years with Bob Pritchard, Richard Novick, Michael Yarmolinsky, and many others, as well as with present and previous co-workers, especially Søren Molin, Luud Koopes, and Gerhart Wagner. However, none of these should be blamed for the content of this paper.

REFERENCES

1. Andresdottir, V., and M. Masters (1978) Evidence that F'lac replicates asynchronously during the cell cycle of Escherichia coli B/r. Mol. Gen. Genet. 163:205-212.
2. Bazaral, M., and D.R. Helinski (1970) Replication of a bacterial plasmid and an episome in Escherichia coli. Biochemistry 9:399-406.
3. Brady, G., J. Frey, H. Danbara, and K.N. Timmis (1983) Replication control mutations of plasmid R6-5 and their effects on interactions of the RNA-I control element and its target. J. Bact. 154:429-436.
4. Carter, B.J., and M.G. Smith (1970) Intracellular pools of bacteriophage λ deoxyribonucleic acid. J. Mol. Biol. 50:713-718.
5. Cesareni, G., M.A. Muesing, and B. Polisky (1982) Control of ColE1 DNA replication - The rop gene product negatively affects transcription from the replication primer promoter. Proc. Natl. Acad. Sci., USA 79:6513-6517.
6. Chandler, M.L., L. Silver, and L. Caro (1977) Suppression of an Escherichia coli dnaA mutation by the integrated R factor R100.1: Origin of chromosome replication during exponential growth. J. Bact. 131:421-430.
7. Collins, J., and R.H.Pritchard (1973) Relationship between chromosome replication and F'lac episome replication in Escherichia coli. J. Mol. Biol. 78:143-155.
8. Cooper, S., and C.E. Helmstetter (1968) Chromosome replication and the division cycle of Escherichia coli B/r. J. Mol. Biol. 31:519-540.
9. Cress, D.E., and B.C. Kline (1976) Isolation and characterization of Escherichia coli chromosomal mutants affecting plasmid copy number. J. Bact. 125:635-642.
10. Diaz, R., K. Nordström, and W.L. Staudenbauer (1981) Plasmid R1 DNA replication dependent on protein synthesis in cell-free extracts of E. coli. Nature 289:326-328.
11. Engberg, B., and K. Nordström (1975) Replication of R-factor R1 in Escherichia coli K-12 at different growth rates. J. Bact. 123:179-186.
12. Gustafsson, P., K. Nordström, and J.W. Perram (1978) Selection and timing of replication of plasmids R1drd-19 and F'lac in Escherichia coli. Plasmid 1:187-203.
13. Gustafsson, P., and K. Nordström (1980) Control of replication of plasmid R1. Kinetics of replication in shifts between different copy number levels. J. Bact. 141:106-110.
14. Hirose, S., S. Hiraga, and T. Okazaki (1983) Initiation site of deoxyribonucleotide polymerization at the replication origin of the Escherichia coli chromosome. Mol. Gen. Genet. 189:422-431.
15. Hobom, G. (1981) Replication signals in prokaryotic DNA. Curr. Topics Microbiol. Immunol. 94/95:93-142.
16. Inuzuka, M., and D.R. Helinski (1978) Replication of antibiotic resistance plasmid R6K DNA in vitro. Biochemistry 17:2567-2573.

17. Inuzuka, M., and D.R. Helinski (1978) Requirements of a plasmid-encoded protein for replication in vitro of plasmid R6K. Proc. Natl. Acad. Sci., USA 75:5381-5385.
18. Jacob, F., S. Brenner, and F. Cuzin (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329-348.
19. Khan, S.A., S.M. Carleton, and R.P. Novick (1981) Replication of plasmid pT181 DNA in vitro: Requirement for a plasmid-coded product. Proc. Natl. Acad. Sci., USA 78:4902-4906.
20. Kollek, R., W. Oertel, and W. Goebel (1978) Isolation and characterization of the minimal fragment required for autonomous replication of a copy mutant (pKN102) of the antibiotic resistance factor R1. Mol. Gen. Genet. 162:51-58.
21. Kolter, R., and D.R. Helinski (1979) Regulation of initiation of DNA replication. Ann. Rev. Genet. 13:355-392.
22. Kolter, R. (1981) Replication properties of plasmid R6K. Plasmid 5:2-9.
23. Leonard, A.C., J.A. Hucul, and C.E. Helmstetter (1982) Kinetics of minichromosome replication in Escherichia coli B/r. J. Bact. 149:499-507.
24. Light, J., and S. Molin (1981) Replication control functions of plasmid R1 act as inhibitors of expression of a gene required for replication. Mol. Gen. Genet. 184:56-61.
25. Light, J., and S. Molin (1982) The sites of action of the two copy number control functions of plasmid R1. Mol. Gen. Genet. 187:486-493.
26. Light, J., and S. Molin (1983) Post-transcriptional control of expression of the repA gene of plasmid R1 mediated by a small RNA molecule. EMBO J. 2:93-98.
27. Lipinska, B., A. Podhajska, and K. Taylor (1980) Synthesis and decay of λ DNA replication proteins in minicells. Biochem. Biophys. Res. Commun. 92:120-126.
28. Macrina, F.L., G.G. Weatherly, and R. Curtiss III (1974) R6K plasmid replication: Influence of chromosomal genotype in minicell-producing strains of Escherichia coli K-12. J. Bact. 120:1387-1400.
29. Masai, H., Y. Kaziro, and K.-I. Arai (1983) Definition of oriR, the minimum DNA segment essential for initiation of R1 plasmid replication in vitro. Proc. Natl. Acad. Sci., USA 80:6814-6818.
30. Matsubara, K. (1976) Genetic structure and regulation of replication of plasmid λdv. J. Mol. Biol. 102:427-439.
31. Meacock, P.A., and S.N. Cohen (1980) Partitioning of bacterial plasmid during cell division: A cis-acting locus that accomplishes stable plasmid inheritance. Cell 20:529-542.
32. Molin, S., and K. Nordström (1980) Control of plasmid R1 replication: Functions involved in replication, copy number control, incompatibility, and switch-off of replication. J. Bact. 141:111-120.
33. Molin, S., P. Stougaard, J. Light, M. Nordström, and K.

Nordström (1981) Isolation and characterization of new copy mutants of plasmid R1 and identification of a polypeptide involved in copy number control. Mol. Gen. Genet. 181:123-130.

34. Morris, C.F., H. Hashimoto, S. Mickel, and R. Rownd (1974) Round of replication mutant of a drug resistance factor. J. Bact. 118:855-866.
35. Muesing, M., J. Tamm, H.M. Shepard, and B. Polisky (1981) A single base-pair alteration is responsible for the DNA overproduction phenotype of a plasmid copy-number mutant. Cell 24:235-242.
36. Murotsu, T., and K. Matsubara (1980) Role of an autorepression system in the control of λdv plasmid copy number and incompatibility. Mol. Gen. Genet. 179:509-519.
37. Nishimura, Y., L. Caro, C.M. Berg, and Y. Hirota (1971) Chromosome replication in Escherichia coli. IV. Control of chromosome replication and cell division by an integrated episome. J. Mol. Biol. 55:441-456.
38. Nordström, K. (1983) Replication of plasmid R1: Meselson-Stahl density shift experiments revisited. Plasmid 9:218-221.
39. Nordström, K. (1983) Control of plasmid replication: A synthesis occasioned by the recent EMBO Workshop "Replication of prokaryotic DNA," held at de Eemhof, The Netherlands, May 1982. Plasmid 9:1-7.
40. Nordström, K., L.C. Ingram, and A. Lundbäck (1972) Mutations in R-factors of Escherichia coli causing an increased number of R-factor copies per chromosome. J. Bact. 110:562-569.
41. Nordström, K., S. Molin, and H. Aagaard-Hansen (1980) Partitioning of plasmid R1 in Escherichia coli. I. Kinetics of loss of plasmid derivatives deleted of the par region. Plasmid 4:215-227.
42. Nordström, K., S. Molin, and H. Aagaard-Hansen (1980) Partitioning of plasmid R1 in Escherichia coli. II. Incompatibility properties of the partitioning system. Plasmid 4:332-349.
43. Nordström, K., S. Molin, and J. Light (1984) Control of replication of bacteriol plasmids: Genetics, molecular biology, and physiology of the plasmid R1 system. Plasmid (in press).
44. Novick, R.P., and F.C. Hoppensteadt (1978) On plasmid incompatibility. Plasmid 1:421-434.
45. Ogura, T., and S. Hiraga (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci., USA 80:4784-4788.
46. Pritchard, R.H. (1968) Control of DNA synthesis in bacteria. Heredity 23:472.
47. Pritchard. R.H. (1969) Control of replication of genetic material in bacteria. In Bacterial Episomes and Plasmids. A Ciba Foundation Symposium, G.E.W. Wolstenholme and M. O'Conner, eds. J. & A. Churchill Ltd., London, pp. 65-74.
48. Pritchard, R.H., P.T. Barth, and J. Collins (1969) Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19:263-297.

49. Riise, E., P. Stougaard, B. Bindslev, K. Nordström, and S. Molin (1982) Molecular cloning and functional characterization of the copy number control gene copB from plasmid R1. J. Bact. 151:1136-1145.
50. Rasmussen, K.V., T. Atlung, G. Kerszman, G.E. Hansen, and F.G. Hansen (1983) Conditional change of DNA replication control in an RNA polymerase mutant of Escherichia coli. J. Bact. 154:443-454.
51. Rosen, J., H. Ohtsubo, and E. Ohtsubo (1979) The nucleotide sequence of the region surrounding the replication origin of an R100 resistance factor derivative. Mol. Gen. Genet. 171:287-293.
52. Rosen, J., T. Ryder, H. Ohtsuba, and E. Ohtsuba (1981) Role of RNA transcripts in replication incompatibility and copy number control of antibiotic resistance plasmid derivatives. Nature 290:794-797.
53. Rownd, R.H. (1969) Replication of a bacterial episome under relaxed control. J. Mol. Biol. 44:387-402.
54. Rownd, R.H., A.M. Easton, and P. Sampathkumar (1981) Copy number control and incompatibility of incFII R plasmids. In Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, R.C. Clowes, S.B. Levy, and E.L. Koenig, eds. Plenum, New York, pp. 303-315.
55. Schafferman, A., R. Kolter, D. Stalker, and D. Helinski (1982) Plasmid R6K DNA replication. III. Regulatory properties of the initiation protein. J. Mol. Biol. 161:57-76.
56. Simons, R.W., and N. Kleckner (1983) Translational control of IS10 transposon. Cell 34:683-691.
57. Søgard-Andersen, L., L.A. Rokeach, and S. Molin (1984) Regulated expression of a gene important for replication of plasmid F in E. coli EMBO J. 3:257-262.
58. Sompayrac, L., and O. Maaløe (1973) Autorepressor model for control of DNA replication. Nature (London) New Biol. 241:133-135.
59. Stalker, D., M. Filutowicz, and D. Helinski (1983) Release of initiation control by a mutational alteration in the R6K π protein required for plasmid DNA replication. Proc. Nat. Acad. Sci., USA 80:5500-5504.
60. Stalker, D.M., R. Kolter, and D.R. Helinski (1979) Nucleotide sequence of an origin of replication of the antibodic resistance plasmid R6K. Proc. Natl. Acad. Sci., USA 76:1150-1154.
61. Stalker, D.M., C.M. Thomas, and D.R. Helinski (1981) Nucleotide sequence of the region of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:8-12.
62. Stougaard, P., S. Molin, and K. Nordström (1981) RNAs involved in copy-number control and incompatibility of plasmid R1. Proc. Natl. Acad. Sci., USA 78:6008-6012.
63. Tanaka, M., H. Ohmori, and S. Hiraga (1983) A novel type of E. coli mutants with increased chromosomal copy number. Mol. Gen. Genet. 192:51-60.

64. Thomas, C.T. (1981) Molecular genetics of broad host range plasmid RK2. Plasmid 5:10-19.
65. Timmis, K.N., S.N. Cohen, and F.C. Cabello (1978) DNA cloning and the analysis of plasmid structure and function. Progr. Mol. Subcell Biol. 6:1-58.
66. Timmis, K., and U. Winkler (1973) Gene dosage studies with pleiotrophic mutants of Serratia marcescens superactive in the synthesis of marcescin A and certain other exocellular proteins. Mol. Gen. Genet. 124:207-217.
67. Tolun, A., and D.R. Helinski (1981) Direct repeats of the F plasmid incC region express F incompatibility. Cell 24:687-694.
68. Tomizawa, J., H. Ohmori, and R.E. Bird (1977) Origin of replication of colicin El plasmid DNA. Proc. Natl. Acad. Sci., USA 74:1865-1869.
69. Tomizawa, J.-L., T. Itoh, G. Selzer, and T. Som (1981) Inhibition of ColEl RNA primer formation by a plasmid-specified small RNA. Proc. Natl. Acad. Sci., USA 78:1421-1425.
70. Tsurimoto, T., and K. Matsubara (1981) Purification of bacteriophage λ0 protein that specifically binds to the origin of replication. Mol. Gen. Genet. 181:325-331.
71. Tsurimoto, T., and K. Matsubara (1981) Interaction of bacteriophage lambda 0 protein with the lambda origin sequence. ICN-UCLA Symp. Mol. Cell Biol. 22:263-275.
72. Tsutsui, H., and K. Matsubara (1981) Replication control and switch-off function as observed with a mini-F factor plasmid. J. Bact. 147:509-516.
73. Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsubara (1983) Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bact. 155:337-344.
74. Uhlin, B.E., and K. Nordström (1975) Plasmid incompatibility and control of replication: Copy mutants of the R-factor Rl in Escherichia coli Kd-12. J. Bact. 124:641-649.
75. Uhlin, B.E., and K. Nordström (1978) A runaway-replication mutant of plasmid Rldrd-19: Temperature-dependent loss of copy number control. Mol. Gen. Genet. 165:167-179.
76. Willetts, N., and R. Skurrey (1980) The conjugation system of F-like plasmids. Ann. Rev. Genet. 14:41-76.
77. Young, E.T., and R.L. Sinsheimer (1967) Vegetative bacteriophage DNA. II. Physical characterization and replication. J. Mol. Biol. 30:165-200.

THE PARTITION FUNCTIONS OF P1, P7, AND F MINIPLASMIDS

Stuart Austin and Ann Abeles

Laboratory of Genetics and Recombinant DNA
Litton Bionetics, Inc., Basic Research Program
National Cancer Institute, Frederick Cancer
Research Facility
P.O. Box B
Frederick, Maryland 21701

ABSTRACT

The partition regions of P1, P7, and F miniplasmids are discrete DNA sequences of about 3 kb in length that will promote accurate partition of hybrid plasmids independent of the source of replication functions or the position or orientation of the elements.

Each of the par regions seems to be very similarly organized, with open reading frames for essential proteins and a terminal site which appears to be analogous to the centromere of eukaryotic cells. When cloned, these terminal sites exert incompatibility against their respective parent plasmids presumably because they can compete with the parent plasmids as substrates for partition.

We have determined the complete DNA sequence of the P1 par region. In addition to the open reading frame for the essential parA protein (42-44 kd), the region contains a second open reading frame which could encode a 38-kd protein. The 2 large open reading frames appear to form an operon that is negatively regulated from a site adjacent to the promoter and responds to the par gene products in trans. Both this site and the downstream "centromere" site, incB, contain blocks of extremely AT-rich sequences, which are postulated to be binding sites for par proteins. The incB and upstream AT-rich regions both contain 20-bp imperfect inverted repeats. Further downstream from the minimal incB sequence (172 bp) lies an additional region which is essential for partition.

The further analysis of the P1 par region should be greatly facilitated by the finding that it can function in cis to stabilize pBR322 vectors under conditions where the copy number of pBR322 is reduced.

INTRODUCTION

Of the low copy-number plasmids of Escherichia coli, the sex-factor F and the plasmid prophage of bacteriophage P1 are among the most stringently controlled (9). At slow growth rates, the extreme case of having only 2 copies per dividing cell seems to be achieved. The plasmid prophage of bacteriophage P7 is closely related to P1, but differs in key plasmid maintenance functions. It is maintained at similar copy number to F and P1 as judged by the quantity of plasmid DNA detected by gel electrophoresis or hybridization techniques (Austin, unpub. data). Loss of these plasmids from growing cells is very rare; P1 being lost in less than 1 in 10^5 cell division events (5). In addition to a stringent, low-error system for replication control, these plasmids must be substrates for a highly accurate partition system which insures that each daughter cell receives a plasmid copy. It is generally assumed that the partition system promotes attachment of the daughter DNAs to some cellular structure, probably the cell envelope, which pushes or pulls the DNA copies into opposite halves of the dividing cell. These plasmids should, therefore, contain information for their own partitioning which is analogous to the centromere of eukaryotic chromosomes. Three important criteria have been used to establish the existence of such sequences. Firstly, mutation or deletion of the sequences allows replication to continue but causes instability of the plasmid due to random distribution of plasmid copies to daughter cells. Secondly, the necessary sequences can stabilize foreign replicons which, themselves, lack an autochthanous partition system. Thirdly, the essential cis-acting sequences which constitute the recognition sites for partition act as incompatibility determinants because, when cloned in a suitable vector, they compete for partitioning with a resident plasmid, displacing it from the growing population.

Random distribution of plasmids with such low copy number leads to a very high rate of loss, approaching 25% per cell division event at slow growth rates. Thus, plasmid mutants which are partition-defective have a very strong phenotype. Partition mutants can readily be distinguished from mutants defective in accessory mechanisms which aid partition such as those which resolve plasmid dimers (5) or block cell division when errors in replication or partitioning occur (8).

As the 3 above-mentioned unit-copy plasmids are all large and contain much information which is dispensable for plasmid maintenance, a necessary first step for their analysis is to construct

miniplasmids which lack inessential information. Studies on F (2,7) and P1 (2) miniplasmids have shown that the information required for partition of these plasmids is contained in a region of approximately 3 kb which is adjacent to, but separable from, the replication functions. Figure 1 illustrates the general organization of the plasmid maintenance regions of F and P1 as cloned in a λΔatt vector. Each encodes proteins essential for partition, and contains a cis-

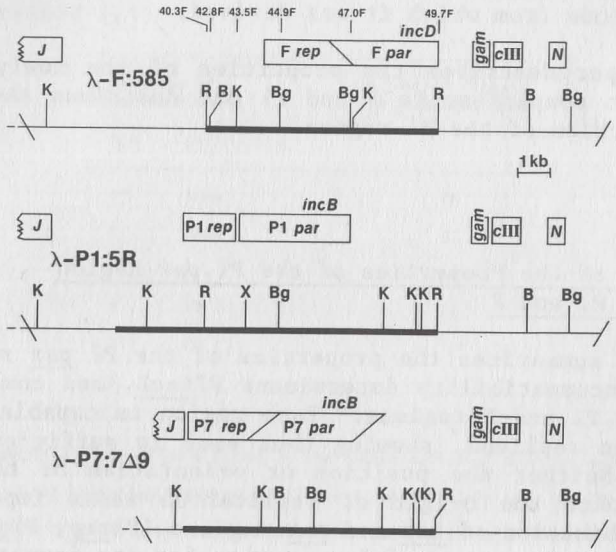


Fig. 1. Physical maps of λ clones containing F, P1, and P7 plasmid maintenance regions. The λimm 21b538 phages lack the att attachment site, and therefore, are dependent on the plasmid maintenance functions to form lysogens. λ-F:585 and λ-P1:5R were constructed as previously described (2). λ-P7:7Δ9 was constructed by cloning EcoR1 fragments of P7 DNA into the unique EcoRI site of the λ vector. One clone, λ-P7:7, contained an 11.8-kb fragment which was substantially homologous to the EcoR1 fragments 8 and 5 of P1 and contained parts of the P7 BamHI fragments 1 and 3 (6). Deletion mutants which delete into the inserted DNA were selected as previously described (5), and were mapped by restriction digest and gel electrophoresis. λ-P7:7Δ9 is one of these which was able to lysogenize as a stable unit-copy plasmid. The P7 insert is oriented in the opposite direction to the standard P7 map (6). The existence of the KpnI site in parenthesis is not certain. Both the regions to the left and right of the P7 par region are substantially homologous to the corresponding P1 regions. R = EcoRI; B = BamHI; Bg = BglII; K = KpnI; X = XhoI.

acting site which is thought to be the recognition site for partition. When cloned in suitable vectors, these sites exert incompatibility against their parental plasmids by competing efficiently as substrates for partition. We have recently cloned the plasmid maintenance region from P7. Initial studies show that it contains a partition region which seems to be similarly organized to P1 and F par regions (Fig. 1). Each of the 3 par regions are different in specificity as none of the cloned incompatibility determinants (partition sites) is effective at competing for partition with plasmids other than those from which it was derived.

This paper describes the properties of the newly isolated P7 par region in comparison to F and P1 and describes the fine-structure and function of the P1 region.

RESULTS

A Comparison of the Properties of the P1 par Region with that of P1 and F

Table 1 summarizes the properties of the P7 par region and its associated incompatibility determinant P7incB, and compares them to those of the P1 and F regions. Each region is capable of stabilizing a foreign replicon, showing that each is sufficient to promote partition. Neither the position or orientation of the par region with respect to the origin of replication seems important. Note that one combination of rep and par regions (P1rep, P7par) could not be isolated despite a powerful selection for its formation. We feel that this may reflect some inappropriate juxtaposition of sequences in this one particular construct rather than a replicon specificity for P7par. Otherwise, all possible combinations give rise to stable composites. The par regions, although functionally equivalent, show a complete species specificity of mechanism as they do not compete with each other for partition, and the cloned incompatibility determinants are only effective against the plasmid from which they were derived (Tab. 2).

The Complete DNA Sequence of the P1 par Region

We have determined the DNA sequence of 6 kb of the P1 plasmid maintenance region which includes both the rep and par sequences. The open reading frame for the 42-44 kd par protein (3) is present in the position predicted (parA; Fig. 1). An additional large open reading frame is also present (parB) which could encode a 34-kd protein. Although we have not been able to detect a protein product for this open reading frame by the maxicell techniques (10), it seems likely that such a produce is made as the potential codon usage of parB is normal with respect to parA and repA genes of P1 (data not shown, Ref. 1). The parB open reading frame is read in

Tab. 1. Composite plasmids were formed by the recombination of deletion mutants of 2 of the λminiplasmids shown in Fig. 1, which retain only the rep or par regions of those plasmids (2). The composites contain the sequences from both parents, bringing different combinations of rep and par sequences together. The strains carrying the composites were then made recombination defective to prevent composite breakdown, and the stability of plasmid maintenance without selection determined as previously described (2).

Genotype of Composite Plasmid		% Loss of Plasmid in 25 Generations
rep	par	
P1	None	96
F	None	97
P1	F	6
F	P1	1
P1	P7	-*
F	P7	2

Composite plasmids were formed by the recombination of deletion mutants of two of the λminiplasmids shown in Figure 1, which retain only the rep or par regions of those plasmids (see Austin and Abeles, 1983a). The composites contain the sequences from both parents, bringing different combinations of rep and par sequences together. The strains carrying the composites were then made recombination defective to prevent composite breakdown, and the stability of plasmid maintenance without selection determined as previously described (Austin and Abeles, 1983a).

*No composites were formed.

the same direction as parA and lies between parA and incB. Thus, the general organization of the P1 par region much resembles that of F (Fig. 2; Ref. 7,12).

We have also sequenced the par regions of the par17 amber mutant. The par17 mutation, which blocks partition (3), is caused by a CAG to TAG change at codon 180 of the parA open reading frame. This generates the 16-kd nonsense fragment of the protein as found previously in maxicell experiments (3).

Tab. 2. Strain N100 recA carried incompatibility determinates from the par regions of F, P1, or P7 in a pBR322 vector. The λ-miniplasmids were introduced by infection, selecting lysogens with clear selector phages (2). The lysogenic colonies were picked and allowed to grow for 25 generations without selection. Isolated colonies formed by these cells were tested for the presence of the λ-miniplasmids by scoring for λ-immunity. No loss of the pBR322 derivatives was seen during the experiment.

	Loss of λ Plasmid After 25 Generations in Cells Transformed With			
Resident Plasmid	pBR322	pALA17 (P1incB)	pALA32 (FincD)	pALA152 (P7incB)
λ-P1:5R	8	98	5	2
λ-F:583	<1	<1	85	1
λ-P7:7Δ9	3	1	7	97

Strain N100 recA carried incompatibility determinates from the par regions of F, P1 or P7 in à pBR322 vector. The λ-miniplasmids were introduced by infection, selecting lysogens with clear selector phages (Austin and Abeles, 1983a). The lysogenic colonies were picked and allowed to grow for 25 generations without selection. Isolated colonies formed by these cells were tested for the presence of the λ-miniplasmids by scoring for λ-immunity. No loss of the pBR322 derivatives was seen during the experiment.

The Par Region of P1 Can Stabilize pBR322

High copy-number plasmids should not need a sophisticated partition mechanism, as random distribution would be effective in ensuring partition. However, if the copy number is reduced sufficiently, random distribution should be ineffective. We have found that the copy number of pBR322 is drastically reduced in a polAts strain at 30°C due to the relatively poor activity of the polA gene product which is essential for replication of plasmids of this type (11). pBR322 is very unstable under these conditions. Derivatives of pBR322 that carry the P1 par region are considerably more stable (Fig. 2), suggesting that pBR322 does not contain a partition system of its own, but can be segregated by the P1 system. The P1parA-mutation par17 blocks the stabilization. As par17 is an amber mutation in the parA open reading frame, this result confirms that the par system is being used by the plasmid under these conditions. Using this assay for par function in pBR322 constructs, we have been able to localize the minimal par region to a HindIII-HpaI DNA fragment of 3.3 kb. The necessary material extends

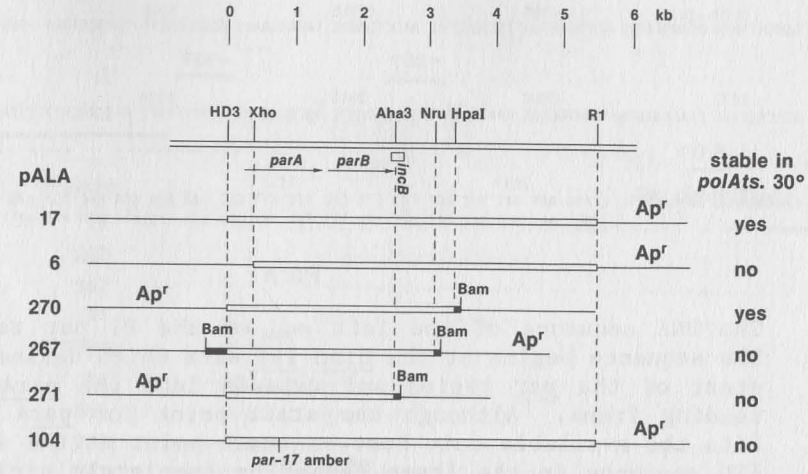


Fig. 2. All plasmids were constructed by cloning fragments into pBR322 vectors. The fragments were derived from λ-P1:5R or from λ-P1:5R fragments cloned into M13Mp10 or M13Mp11 vectors. Single lines = pBR322 DNA (part of). Open boxes = P1 DNA. Closed boxes = linkers derived from M13 or pBR322 vectors. The polA12ts strain was transformed with the plasmid DNA's at 30° selecting for ampicillin resistance. Stability was measured by scoring the ampicillin resistance of the cells after 10 generations of unselected growth in broth. Typically, stable plasmids are lost in each 5% of the cells, whereas unstable ones are lost in 95%. At 40°, all plasmids were lost for <98% of the cells in 10 generations. HD3 = HindIII; Xho = XhoI; Aha3 = AhaIII; Bam = BamIII.

further to the right than previously estimated (Fig. 1; Ref. 2) and includes the parA and parB open reading frames, the incB sequence, and some additional sequences to the right of incB. Plasmids which lack the amino-terminal of the parA gene, or incB, or the region to the right of it are no more stably maintained than pBR322 itself (Fig. 2).

Regulation of the Par Proteins

Although the parB open reading frame appears to be used, inspection of the DNA sequence shows no obvious promoter site immediately upstream of it, and the distance between parA and the probable translation start point for parB is only 16. It seems probable that parA and parB are contranscribed from a promoter upstream of parA. Figure 3 shows the DNA sequence adjacent to the -10 region of the putative promoter and occupying the space between this promoter and the likely Shine and Dalgarno (ribosome binding site) suggested that

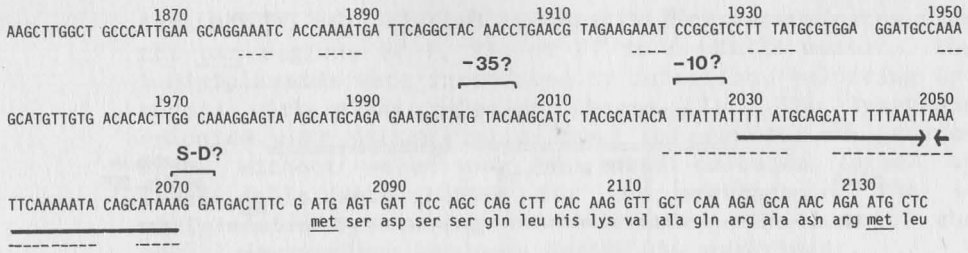


Fig. 3. The DNA sequence of the left end of the P1 par region. The sequence begins at the Hind III site which defines the start of the par region and extends into the parA open reading frame. Although the start point for parA shown fits the available data best, a start point at the second ATG sequence in the frame cannot be completely excluded. The dashed underlines show runs of 4 or more A or T bases. The 20 bp inverted repeat sequence requires 2 mismatches and a single base loop-out to give maximum complementarity.

some regulatory element acts here. In order to test this, a plasmid was constructed which has the first 53 codons of parA fused in frame with the β-galactosidase gene. This plasmid produces a Lac$^+$ fusion protein under the control of the par promoter. We have shown that the production of the hybrid β-galactosidase activity is strongly downregulated by the presence of an intact P1 par region in trans (data not shown). Initial experiments suggest that both parA and parB products are required for this downregulation. Thus, the par operon appears to be subject to a novel type of autoregulation for which both products are required to act as the repressor. Our observations on the properties of amber mutants with lesions in the genes for the two F par proteins suggested the existence of the same type of regulation in F (4).

The Nature of incB

The P1 incB locus exerts a strong incompatibility effect when cloned in a pBR322 vector (Tab. 2). By subcloning fragments from this region, we have located a sequence sufficient to exert this phenotype to a 175-bp TaqI-HincII fragment (Fig. 4). The sequence of this fragment is shown in Fig. 5. It contains the end of the parB open reading frame and a downstream region which contains an imperfect 20 inverted repeat sequence. This sequence is AT-rich. Bracketing this feature are 2 highly AT-rich sequences, the right, most of which has 17 contiguous AT pairs with 2 blocks of 7As in the same strand.

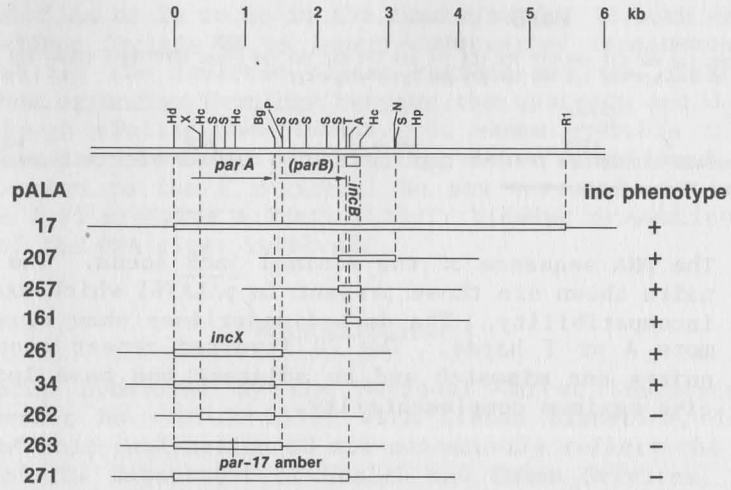


Fig. 4. All plasmids were derived from pBR322 vectors. The presence of incompatibility determinants were determined by: (a) introducing λ-P1:5R under selection to N100 recA cells carrying the plasmid and then measuring the loss of λP1:5R under nonselective conditions, or (b) transforming established λ-P1:5R lysogens with the pBR322 derived plasmids and scoring loss of λ-P1:5R under nonselective conditions. With inc$^+$ clones, λ-P1:5R was very unstably maintained in both tests, whereas a λ-P7 or λ-F miniplasmid was stably maintained. Hd = HindIII; Hc = HincII; Hp = HpaI; A = AhaIII; Bg = BglII; RI = EcoRI; X = XhoI; P = PvuI; N = NruI; S = Sau3A; T = TaqI. (All other TaqI sites not shown. All 4 base recognitions between HpaI and EcoRI not shown).

A Second Incompatibility Locus Due to Overproduction of parA

During our efforts to locate incB, we discovered a second pBR322 cloned par fragment which could exert incompatibility specifically against P1 (Fig. 4). This incompatibility is due to parA protein synthesis from the fragment as it is entirely abolished by the parA17 amber mutation and requires an intact amino-terminus of the parA open reading frame (Fig. 4). It seems probable that such clones overproduce parA protein due to lack of a parB on the high copy plasmid, a condition which appears to release the autoregulation of the operon. An excess or imbalance of parA protein relative to other components appears to interfere with proper partition.

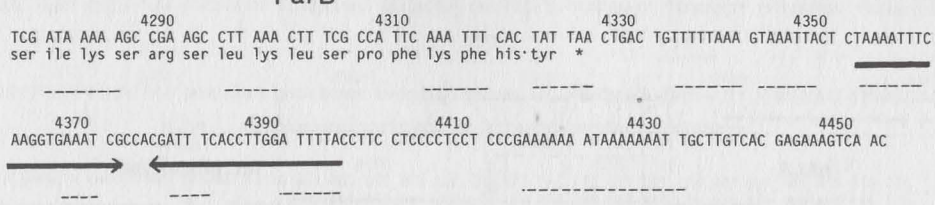


Fig. 5. The DNA sequence of the minimal incB locus. The 175 base pairs shown are those present in pALA161 which exerts incB incompatibility. The dashed underlines show runs of 4 or more A or T bases. The 20 inverted repeat sequence requires one mismatch and an adjacent one base loop-out to give maximum complementarity.

CONCLUSIONS

The partition regions of P1, F, and P7 seem to be very similar in their function and general organization. However, each system appears to specify a unique pathway for partition as none exerts incompatibility against the other types. The specificity is reflected in the specificity of the par region components. Neither F nor P7 encoded par proteins can complement P1 parA mutants (data not shown), and the inc regions of at least F and P1 show no homology as judged by Southern Blot hybridization (Austin, unpub. data). A ca. 2-kb region of nonhomology between P1 and P7 (nonhomology E, Ref. 13) appears to correspond approximately to the position of the P1 and P7 par regions (this paper; Ref. 6).

The individual par regions contain all the necessary plasmid-specified information to promote partition. This allows them to function in the context of foreign replicons. The observation that P1 par can stabilize pBR322 shows that par has no specificity with respect to replication strategy. P1 will, presumably, stabilize any replicon as long as copy number is reduced to the point where specific partition becomes limiting on stability.

It seems probable that par proteins promote partition by binding to the DNA at the inc site, and to the cell envelope to form a specific partition complex. It appears that the concentration of par proteins in the cell is critical for this to occur. Their synthesis appears to be tightly autoregulated in P1, and overproduction of parA protein blocks specific partition. Autoregulation of F par proteins also seems to occur. We would, therefore, expect par proteins to bind to 2 distant regions within the par sequences; in the vicinity of the promoter for par and at the putative centromere site. A striking feature of the DNA sequence in these 2 regions is the presence of highly AT-rich runs of bases with a strong tendency

for blocks of As or Ts to be in the same strand. In both cases, the AT-rich regions include 20 bp imperfect inverted repeats spaced 1-3 apart. Neither the inverted repeat regions nor the other AT-rich regions show extensive homology between the upstream and downstream loci, although similarities abound. It seems probable that these features are important for par binding, as we have found no other features common to the 2 regions. We are currently attempting to purify the 2 P1 proteins and study their binding properties and the identity of the DNA sites involved.

ACKNOWLEDGMENTS

Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-23909 with Litton Bionetics, Inc. The contents of this publication do not necessarily reflect the views of policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

1. Abeles, A., K. Snyder, and D. Chattoraj (1984) P1 plasmid replication: Replicon Structure. J. Mol. Biol. 173:307-324.
2. Austin, S., and A. Abeles (1983a) The partition of unit-copy miniplasmids to daughter cells. I. P1 and F miniplasmids contain discrete, interchangeable sequences sufficient to promote equipartition. J. Mol. Biol. 169:353-372.
3. Austin, S., and A. Abeles (1983b) The partition of unit-copy miniplasmids to daughter cells. II. The partition region of miniplasmid P1 encodes an essential protein and a centromere-like site at which it acts. J. Mol. Biol. 169:373-387.
4. Austin, S., and A. Wierzbicki (1983) Two mini-F encoded proteins are essential for equipartition. Plasmid 10:73-81.
5. Austin, S., M. Ziese, and N. Sternberg (1981) a novel role for site-specific recombination in maintenance of bacterial replicons. Cell 25:729-736.
6. Iida, S., and W. Arber (1979) Multiple physical differences in the genome structure of functionally related bacteriohage P1 and P7. Mol. Gen. Genet. 173:249-261.
7. Ogura, T., and S. Hiraga (1983a) Partition mechanism of F plasmid: Two plasmid gene-encoded products and a cis-acting region are involved in partition. Cell 32:351-360.
8. Ogura, T., and S. Hiraga (1983b) Mini-F plasmid genes that couple host division to plasmid proliferation. Proc. Natl. Acad. Sci., USA 80:4784-4788.
9. Prentki, P., M. Chandler, and L. Caro (1977) Replication of the prophage P1 during the cell-cycle of Escherichia coli. Mol. Gen. Genet. 152:71-76.

10. Sancar, A., A. Hack, and W. Rupp (1979) Simple method for the identification of plasmid-coded proteins. J. Bact. 137:692-693.
11. Kinsbury, D., and D. Helinski (1970) DNA polymerase as a requirement for the maintenance of the bacterial plasmid colicinogenic factor El. Biochem. Biophys. Res. Commun. 41:1538-1544.
12. Komai, N., T Nishizewa, Y. Hayakawa, P. Murotsu, and K. Matsubara (1982) Detection and mapping of six miniF-encoded proteins by cloning analysis of dissected miniF segments. Mol. Gen. Genet. 186:193-203.
13. Yun, T., and D. Vapnek (1977) Electron microscopic analysis of bacteriophage P1, phage P1Cm, and phage P7. Determination of genome sizes, sequences, homology, and location of antibiotic resistance determinants. Virology 77:376-385.

GENETIC INTERACTIONS OF BROAD HOST-RANGE PLASMID RK2:

EVIDENCE FOR A COMPLEX REPLICATION REGULON

David H. Figurski,[1] Calvin Young, Helen C. Schreiner,[2] Robert F. Pohlman, David H. Bechhofer, Alice S. Prince,[3] and Thomas F. D'Amico

Department of Microbiology and Cancer Center
College of Physicians & Surgeons
Columbia University
701 West 168th Street
New York, New York 10032

INTRODUCTION

As first recognized by Jacob, Brenner, and Cuzin (13), a successful plasmid must have an efficient replication control system that ensures its stable maintenance by the host. It is now very apparent that bacterial plasmids have evolved an intriguing variety of regulatory strategies to accomplish this (23). However, it is important to realize that for any such system to work effectively, there are certain requirements which concern the plasmid-host interaction. (i) Plasmid genes for replication control must be expressed adequately by the host at the proper time in the cell cycle. (ii) For initiation of replication, the plasmid initiation complex must interact productively with the host's replication apparatus. (iii) Expression of the plasmid replication determinants must be tightly controlled to prevent an unnecessary metabolic burden or potentially lethal titration of host replication components. (iv) The plasmid must interact with host cell components to guarantee segregation of at least one plasmid copy to each daughter cell at division.

---

[1] Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55108; [2] Department of Microbiology, The Public Health Research Institute of the City of New York, 455 First Avenue, New York, New York 10016; and [3] Departments of Medicine and Microbiology, College of Physicians and Surgeons, Columbia University.

For plasmids maintained only by closely related hosts, it is easy to imagine how such efficient plasmid-host relationships could evolve. However, when one superimposes on these requirements the additional property of an extremely broad host range, as seen for plasmids of incompatibility groups P (7,18) and Q (3), it is less clear how stable maintenance is achieved in each host. Obviously all hosts are not equal with respect to plasmid maintenance because no other plasmid group has a host range which includes virtually all gram-negative bacteria.

The molecular basis for this striking capability is not obvious. One may ask if these plasmids have uniquely capitalized on some common feature of gram-negative bacteria. For example, their initiation complexes may interact well with the replication apparatus of any host. Alternatively, these plasmids may encode different functions for different hosts. Another question concerns the ability to express the essential plasmid genes sufficiently well in each host. Have broad host-range plasmids evolved universal promoters which are reasonably functional in any host, or do important genes have multiple promoters? The answers to these basic questions about the mechanism of broad host range are not yet known and they should be interesting because the challenge to these plasmids is greater than that of their narrower host-range counterparts.

We have been studying the IncP plasmid RK2 with the idea that this broad host-range plasmid may reveal novel genetic determinants for replication control. This expectation seems to have been justified because we have found a new class of maintenance determinants. These are not absolutely required for the plasmid to replicate, but they can have a dramatic effect on plasmid replication control.

ESSENTIAL MAINTENANCE DETERMINANTS OF RK2

Plasmid RK2 (Fig. 1) is a 56 kb broad host-range plasmid of the IncP group (12,15). Two plasmid determinants (*oriV* and *trfA*) have been shown to be absolutely required for plasmid replication. *OriV* is the origin of replication, originally mapped to coordinate 12' of the physical map by electron microscopy of replicative intermediates (16). *OriV* was subsequently shown to behave as a cis-acting replication element which could be activated in trans by the product of another RK2 gene (8). The gene for this diffusible function was named *trfA* (29) and has been mapped to the 16'-18' region of RK2 (19,29). The ability to activate *oriV* in trans allowed strategies for precise localization and identification of the important nucleotide sequences (26). Those needed for replication involve several direct repeats, some of which exhibit incompatibility with RK2-based replicons (30).

OriV and *trfA* comprise the essential minimum for RK2 replication. Plasmids containing only these 2 determinants are able to

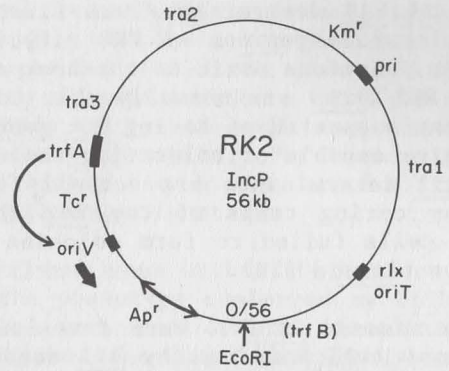


Fig. 1. Basic genetic map of RK2. Presented here are landmark determinants to show the basic organization of the RK2 genetic map. In the text, coordinates indicate the distance in kilobases, clockwise, from the unique EcoRI site and are designated with a prime (e.g., 12'). *OriV* and *trfA* are discussed in the text; the arrow over *oriV* indicates the direction of replication (16). *Tra* indicates regions of genes for conjugal transfer; *pri* is the primase gene (14); *rlx*, relaxation complex nick site (11); Ap^r, Tc^r, and Km^r are genes for resistance to ampicillin, tetracycline, and kanamycin, respectively. The arrowheads in the region of Ap^r show that this is a transposon.

replicate in *Escherichia coli*, *Pseudomonas*, and other gram-negative hosts (22; Schmidhauser and Helinski, pers. comm.). Thus, at least some broad host range capability is encoded within these functions.

QUASI-ESSENTIAL MAINTENANCE DETERMINANTS

We have found that RK2 encodes a second class of maintenance determinants which we call "quasi-essential." This class consists of genes lethal to the host (*kil* genes) and their controlling elements (*kor* genes). Whether one of these genes is essential depends on which other genes are present in the plasmid. Our studies have revealed the following: (i) *kor* genes are essential whenever the corresponding *kil* genes are present on the plasmid; and (ii) a *kil* gene may be essential for plasmid replication when the appropriate *kor* gene is present. Thus, loss of either a *kil* determinant *or* a *kor* determinant will have serious consequences for RK2 maintenance. However, it is possible to lose both because an *oriV*$^+$ *trfA*$^+$ basic replicon can be functional in the absence of *kil* and *kor* genes. Thus, these genes are considered essential only when the corresponding partner is still present in the plasmid.

The existence of kil determinants was first indicated by our inability to clone certain regions of RK2 (10). We reasoned that these regions encode functions toxic to the host cell (kil) and that other functions of RK2 (kor) are normally able to control them. By first cloning regions suspected of having kor genes, we were able to construct host strains capable of tolerating the cloned kil determinants. That the kil determinants are actually lethal to the host was demonstrated by curing cells of the kor$^+$ helper plasmid and showing that these cells failed to form colonies in the absence of selection for either plasmid (10).

Thus, new determinants of RK2 were revealed by 2 unambiguous phenotypes: (i) host cell killing (by kil determinants) and (ii) prevention of host death (by kor determinants). These phenotypes allowed us to locate three separate kil determinants (kilA, kilB, and kilC), each of which is controlled by the product of a specific kor gene (korA, korB, and korC). Their relative positions on the RK2 physical map are shown in Fig. 2.

kilB1 AND THE QUESTION OF trfB

Earlier studies with miniderivatives of RK2 suggested that, in addition to oriV and trfA, a third region (trfB) is required for replication (29). The trfB region was curious because in certain plasmids it seems to be essential (27,29), whereas in others it is clearly dispensable (27). After finding that kilB maps near trfA

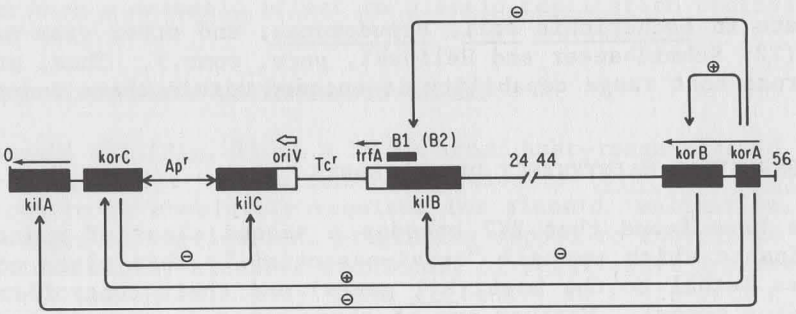


Fig. 2. The kil and kor determinants of RK2 and their interactions. This linear representation of the RK2 map begins at the EcoRI site. Only the relevant regions are presented; the 20-kb segment between 24' and 44' is not shown. Interactions are depicted with "+" indicating a positive effect or "-", a negative effect. Open arrow over oriV shows direction of replication. Direction of transcription for trfA (25), kilA (Young, Prince, and Figurski, in prep.) and korA-korB (4; Bechhofer and Figurski, in prep.) is indicated by an arrow overhead.

and that its control gene (korB) maps near trfB, we proposed that the trfB region actually encodes a kor gene required only when a kil gene near trfA is present (10). We proved this by demonstrating that a trfB-requiring plasmid encodes a kil-like determinant immediately adjacent to trfA (20). As predicted, this kil is controlled by a function from the trfB region. Furthermore, plasmids defective in this kil do replicate, but do not require trfB. Thus, trfB is not an essential replication function.

This kil determinant maps within the kilB region and very likely contributes to the phenotype conferred by kilB. However, it can be regarded as only a component of kilB because it is controlled by the trfB region, whereas the complete kilB is not. We named this component "kilB1." The corresponding control gene was originally named "korB1," but we have since found that korA is sufficient for control of kilB1 (see below). Clearly another determinant of the kilB region is responsible for the requirement for korB, and we have reserved the designation "kilB2" for this component.

[In another study, Smith and Thomas (24) found a kil-like determinant in the same region and named it "kilD," with "korD" as the control gene in trfB. KilB1 and kilD are very likely the same, as are korA and korD.]

In summary, the kilB region has 2 distinct kil determinants: kilB1 (whose existence is verified) and kilB2 (whose existence is deduced). KilB1 is adjacent to trfA and can be controlled by korA, which maps in the region designated trfB. KilB2 is on the other side of kilB1, and together they require more than korA. This additional determinant is korB, which maps next to korA, but extends beyond the trfB region. These relationships are shown in Fig. 2.

STRUCTURE OF korA

By subcloning and manipulation of DNA fragments from the korA-korB region, we mapped the korA gene to the region between coordinates 55.6' and 55.2' (4). Transcription proceeds from 55.6' to 55.2' as determined by promoter replacement experiments and by hybridization analysis with korA-specific RNAs synthesized in vivo. The nucleotide sequence of this region revealed one intact open reading frame preceded by a ribosome binding site and a promoter-like structure with strong homology to the -35 and -10 consensus sequences. The product of this open reading frame, as determined by translation of the sequence, should be a polypeptide of 13,000 d. Evidence that this is korA was provided by BAL31-generated deletion mutants which removed nucleotides from the 3' end of the open reading frame. One mutant which gives a KorA$^-$ phenotype (i.e., unable to control kilA) is missing nine carboxy-terminal residues from the putative product. Another mutant which confers a KorA$^-$ phenotype

(i.e., fewer and smaller transformants with kilA) is missing 2 residues (4).

We have also found that this minimal korA-encoding region is able to control kilB1, and furthermore that the korA mutants are also defective in the control of kilB1. Thus, the same kor gene is responsible for negative control of 2 separate kil determinants (Bechhofer, D'Amico, and Figurski, unpub. results).

korA INHIBITS kilA EXPRESSION

The kilA gene is located in the 0'-2.3' region (32). Insertion of a DNA linker at coordinate 2.2' causes complete loss of kilA expression (Young, Prince, and Figurski, in prep.), but expression is restored if another promoter is inserted here (Fig. 3). With the promoter for tetracycline resistance (p_{tet}), the gene is constitutively expressed at a low level; i.e., cells with this plasmid form small colonies. With p_{lac}, kilA is inducible with IPTG. A host cell mutant partially resistant to kilA is also resistant to the killing activity from these promoter fusions.

In contrast to the wild-type kilA gene, kilA fused to p_{tet} or p_{lac} is not affected by korA (Fig. 3). Thus, replacement of the natural kilA expression region has removed sensitivity to korA. To

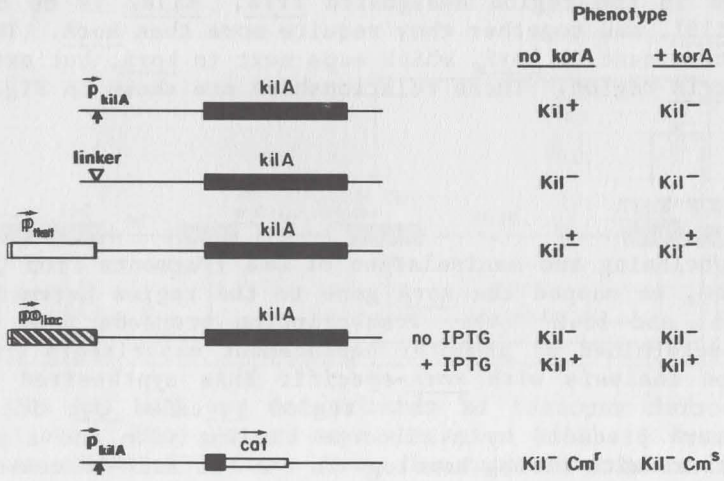


Fig. 3. Analysis of korA control of kilA. This schematic diagram presents constructed variations of the kilA region, as described in the text. The Kil phenotype was assayed by transformation of cells with and without korA. For the cat fusions, transformants were screened for resistance or sensitivity to 50 μg/ml chloramphenicol (Cm).

examine this further, we have fused the structural gene for chloramphenicol transacetylase (cat) to the wild-type kilA promoter region. Plasmids with this fusion confer Cm^r on the cells in the absence of korA, but the cells are Cm^s when korA is present. We conclude that korA negatively controls kilA at the level of gene expression. It is also reasonable to expect that korA controls kilB1 in the same way.

RELATIONSHIP OF korA TO EXPRESSION OF korB

KorA and korB are separate genes because interruption of the SstII site at 54.2' inactivates korB function but not korA (10). However, deletion of the 5' end of korA eliminates expression of both korA and korB. Hybridization analysis of korB-specific RNA showed (i) the direction of transcription is the same as that of korA, and (ii) the korB-specific probe hybridizes to the same RNA bands as does the korA-probe, while these probes show no homology to each other. This evidence suggests that korB is downstream of korA and within the same transcriptional unit (Bechhofer and Figurski, in prep.).

KorA product is not required for korB product to control kilB because it is possible to isolate korB+ plasmids completely deleted for korA (2; D'Amico and Figurski, unpub. data). In one case, we have verified that expression depends on a new promoter fused to korB. This finding implies that, like korA, korB is also able to control the kilB1 component of kilB, and we have preliminary evidence that this is true (Schreiner and Figurski, unpub. data).

Our results suggest that the korA product might be a positive regulator for korB expression. This study involves a Tn76 insertion of the korA-korB region, originally isolated by P. Barth as a host range mutant of RP4 (1), an IncP plasmid identical to RK2 (5,31). Recent studies showed the Tn76 to have inserted between korA and korB (2). This mutation was transferred by marker rescue to a cloned korA-korB segment, and the mutant segment was found to express both KorA+ and KorB+ phenotypes. Because the 15-kb Tn76 insertion is not polar on korB, it seemed likely that a transposon promoter is expressing korB. Indeed, we found that deletion of the Tn76 segment adjacent to the 5' end of korB caused loss of KorB phenotype.

We were therefore surprised to find the following results: (i) deletion of korA from this Tn76 derivative also caused a KorB phenotype; and (ii) addition of korA in trans restored the korB phenotype. Because we already established that korA product is not required for korB control of kilB, we hypothesized that korA product is needed to express the korB gene.

One possibility we considered is activation of a promoter for

korB. However, we found that mutants deleted for korA promoter could not express korB even when korA was supplied in trans. Likewise, in the Tn76-korB fusion, removal of the Tn76 sequences results in a KorB$^-$ phenotype that is no longer reversed by korA in trans. Thus, it seems that korB expression requires a promoter and korA product.

Another possibility we considered is that a transcription termination signal occurs before korB and that korA is required to prevent termination to allow transcription of korB. Thus, regardless of the promoter, korA would be required for expression of korB. In the promoter fusions in which korB is expressed independent of korA, we proposed that the terminator was lost in the construction. Although we have no direct evidence for this model with korB, supporting results have emerged from studies on the interaction of korA and korC.

POSITIVE REGULATION BY korA IN korC EXPRESSION: EVIDENCE FOR ANTITERMINATION

The phenotypes of various insertion and deletion mutants suggested that korC maps in the 3.3'-5.0' region. However, we were surprised to find that this region, when cloned, does not express korC. A second determinant is also required for the KorC$^+$ phenotype, and we found this to be korA (32).

Because we thought korA is required for korB expression by functioning as an antiterminator of transcription, we reasoned that the same mechanism is involved in the korA-dependence of korC. It therefore seemed possible that the putative antitermination function might be bypassed in an E. coli host defective in the termination factor rho (21). Thus, the korC region was tested for its ability to control kilC without korA in rho$^+$ (termination proficient) and rho$^-$ (termination defective) hosts. The results showed clearly that the rho$^-$ mutation bypasses the requirement for korA in the expression of korC (Fig. 4) (32). This is consistent with the interpretation that korA acts as an antiterminator of transcription.

INVOLVEMENT OF kil AND kor DETERMINANTS IN REPLICATION CONTROL

What are the true functions of the kil and kor genes? An indication that they might be important to the IncP group of plasmids first came from our finding that all of 14 different IncP plasmids were positive when tested for korA- and korB-like functions (10). In contrast, plasmids of 19 other incompatibility groups expressed no functions able to control either RK2 kilA or kilB. Thus, the kor genes, and very likely their corresponding kil determinants, are unique features of IncP plasmids. The striking conservation of

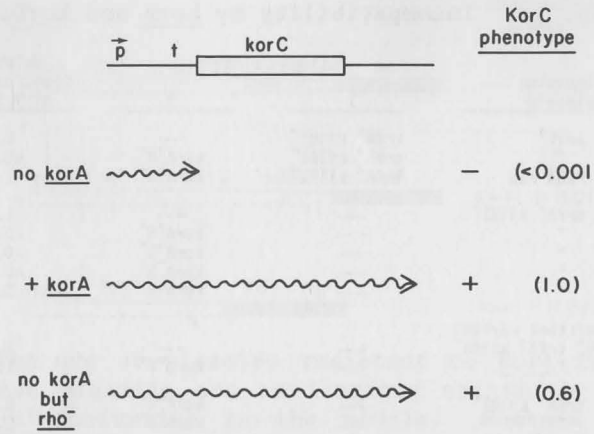


Fig. 4. Effect of a host rho mutation on the korA-dependence of korC. KorC function of this cloned segment was assayed by its ability to control a kilC$^+$ plasmid when korA was present or when the host was rho$^-$. The numbers show relative efficiencies of transformation. A possible mechanism is depicted in the drawing. "p" denotes a promoter; "t", a rho-dependent transcription termination signal.

these functions within the IncP group led us to propose that these genes might be important to plasmid maintenance. We suggested that they were important in non-E. coli hosts because they did not seem to be part of the essential minimum for RK2 replication in E. coli. However, we now have evidence that the action of kil and kor genes can have a profound effect on RK2 replication even in E. coli.

We have observed that both korA and korB exhibit a strong incompatibility towards certain RK2 replicons (Schreiner, Bechhofer, Pohlman, Young, Borden, and Figurski, in prep.). Our analysis of this phenomenon has led us to conclude the following. (i) The incompatibility results from inhibition of replication by the action of korA or korB on the expression of trfA. (ii) The presence or absence of kilB1 in cis to trfA has a striking effect on the sensitivity of the plasmid to the korA- or korB-mediated inhibition of trfA.

These interactions are illustrated by the results in Tab. 1. Whenever a korA$^+$ korB$^+$ plasmid is present in a strain, transformation of that strain by certain RK2 replicons is inhibited at least 100-fold (compare lines 1 and 2; 4 and 5). Non-RK2 replicons are not affected, and in fact linkage of a non-RK2 replicon (e.g., P15A; line 3) to the RK2 plasmid prevents the inhibition. Thus, the interference is at the level of replication, and only plasmids dependent on RK2 oriV for replication are inhibited by the korA-korB region.

Tab. 1. Incompatibility by korA and korB.

	Incoming plasmid	Resident plasmids in host 1	Resident plasmids in host 2	Relative transformation efficiency[a]
1.	oriV$^+$	trfA$^+$ kilB1$^-$	---	1.0
2.	"	trfA$^+$ kilB1$^-$	korA$^+$B$^+$	<0.01
3.	oriV$^+$ P15A rep	trfA$^+$ kilB1$^-$	korA$^+$B$^+$	1.0
4.	oriV$^+$ trfA$^+$ kilB1$^-$	---	---	1.0
5.	"	---	korA$^+$B$^+$	<0.01
6.	"	---	korA$^+$B$^-$	<0.01
7.	"	---	korA$^-$B$^+$	<0.01
8.	"	---	korA$^-$B$^-$	0.5
9.	kor-resistant variant of oriV$^+$ trfA$^+$ kilB1$^-$	---	---	1.0
10.	"	---	korA$^+$B$^+$	1.1
11.	oriV$^+$ trfA$^+$ kilB1$^+$	---	korA$^+$B$^-$	0.3
12.	"	---	korA$^-$B$^+$	0.4

[a] The host strain in each case was MV10 (10). All transformation efficiencies are corrected for variations in competence as measured by transformation with saturating amounts of a non-RK2, compatible plasmid. Variations of less than three-fold are considered insignificant.

Which determinant(s) in the korA-korB region are actually responsible for this incompatibility? Meyer and Hinds (17) have reported an incompatibility determinant, IncP-1(II), in this region. However, the incompatibility observed here is clearly different. The most notable difference is that, unlike the effect of IncP-1(II), the interference by the korA-korB region is suppressed by the addition of another replicon. In fact, we have found that the effect is mediated by the korA and korB genes themselves. Surprisingly, either korA or korB alone will exhibit strong incompatibility (lines 6 and 7). Only when both are missing is the incompatibility lost (line 8). Therefore, korA and korB are each able to inhibit RK2-specific replication.

To begin to answer how this might be accomplished, we sought to identify the targets for korA and korB. The effect of these determinants on the oriV$^+$ trfA$^+$ kilB1$^-$ plasmid used in Tab. 1 is so strong that it was possible to select a plasmid mutant resistant to korA and korB (Tab. 1, line 9 and 10). Analysis of the structure of this mutant has revealed a likely mechanism for korA- and korB-mediated incompatibility (Fig. 5). Because this plasmid has suffered a deletion in the promoter region of trfA and because the plasmid is completely resistant to korA and korB, the deleted segment must encode the targets.

The simplest explanation is that korA and korB products interact with recognition signals in the promoter region of trfA to inhibit expression of the trfA gene. In this way plasmid replication is prevented. In the kor-resistant mutant, the recognition signals for korA and korB products are missing.

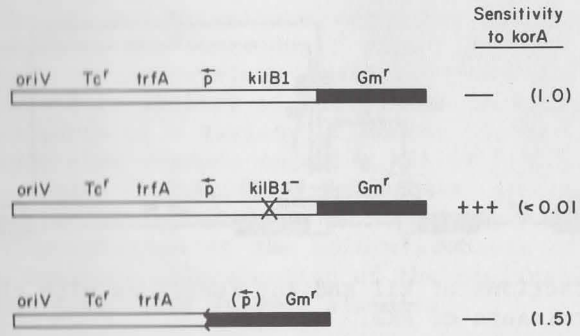


Fig. 5. Structure of plasmids resistant to korA incompatibility. These plasmids are an isogenic set which stems from the kilB1-derivative in the middle. Numbers show relative transformation efficiencies into a korA$^+$ host. In the top plasmid, the kilB1$^-$ allele is replaced with kilB1$^+$. At bottom is the structure of a korA- and korB-resistant mutant. Promoters are indicated with "p" and direction of transcription with an arrow. "(p)" in the bottom derivative suggests that trfA is expressed from a promoter in the Gm^r-encoding region because the known trfA promoter (25) is deleted.

We also noticed that kilB1 has a dramatic effect on the ability of korA and korB to inhibit replication. The test plasmids used in the studies in Tab. 1 contain a kilB1$^-$ allele of a spontaneous Kil$^-$ mutant isolated previously (20). As shown above, these plasmids are highly sensitive to korA and korB incompatibility. However, if the kilB1$^-$ allele is replaced with the wild-type kilB1$^+$ allele, the new plasmid shows little if any sensitivity to korA and korB (Tab. 1, lines 11 and 12). This effect of kilB1$^+$ occurs only in cis; a kilB1$^+$ determinant alone in trans has no effect (data not shown). This is a remarkable result, for it implies that in a korA$^+$ or korB$^+$ plasmid kilB1 should be considered an essential replication determinant. This is, of course, the case for parental RK2.

Thus, we have shown that korA and korB can inhibit RK2-specific replication. The results indicate that korA and korB products inhibit expression of the trfA gene and that this is accomplished by direct interaction with regulatory sequences in the promoter region of trfA. Furthermore, the inhibitory effects of korA and korB are somehow modulated by the kilB1 determinant. These interactions are summarized in Fig. 6.

SUMMARY

The kil and kor genes of RK2 are novel genetic determinants

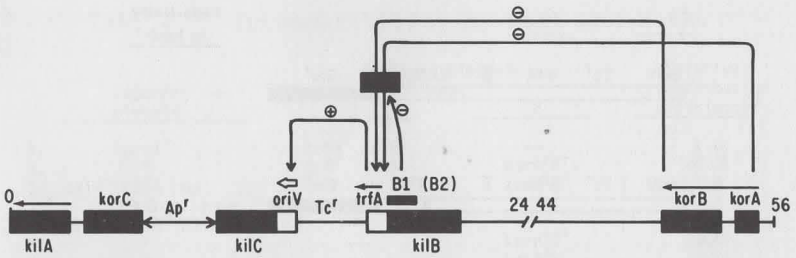


Fig. 6. Interactions of kil and kor functions with the replication determinants of RK2.

which seem to be common to all IncP plasmids. Their presence is easily detected by the strong phenotypes of host cell killing and its prevention. Thus, it has been possible to do extensive genetic analysis of these determinants, and this has revealed an intricate network of interactions. KorA has emerged as the central component in this network because of its multiple functions--both positive and negative.

It is now clear that the kil and kor genes constitute a new class of elements involved in plasmid maintenance. They cannot be considered absolutely essential for replication because it is possible to construct functional RK2 replicons which are devoid of any known kil or kor determinants. Nevertheless, the network of kil and kor interactions has now been shown to include interactions with essential replication determinants, and therefore there is a direct link between the action of these genes and control of plasmid replication (Fig. 7).

Originally we speculated that the kil and kor genes might be important to the broad host range of RK2 (10). We wish to propose

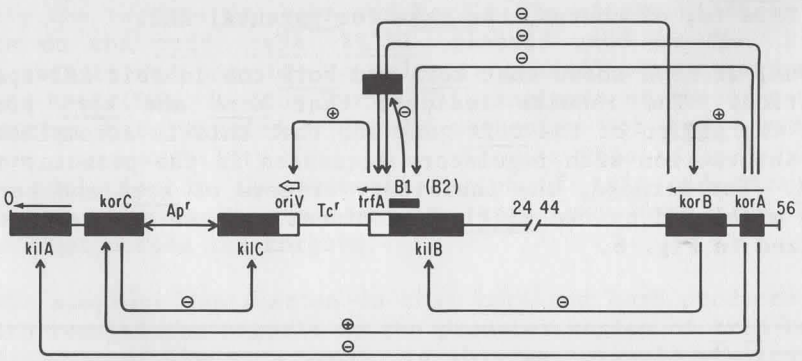


Fig. 7. Summary of the genetic interactions of RK2.

further that the kil and kor network constitutes a replication regulon, and that perhaps the function of this regulon is to ensure expression of trfA at appropriate levels. The complexity of this regulon may reflect an ability of the system to adapt to the intracellular environments of a variety of hosts. Indeed, there is tantalizing evidence that regions encoding kil or kor genes are important to host range (1,2,6,28; Schmidhauser and Helinski, pers. comm.). We are therefore hopeful that the study of these genes and the eventual determination of the molecular basis of their actions will lead to a complete understanding of the replication control and broad host range capability of IncP plasmids.

ACKNOWLEDGMENTS

We deeply appreciate the help of N. Scott, H. Spivak, B. Lynch and numerous students who spent summers or rotations in the lab: L. Babiss, S. Bessim, P. Borden, J. Davis, M. Dimitrov, M. Donnenberg, L. Epstein, P. Freimuth, I. Gelman, P. Gregor, B. Hill, O. Jovanovic, C. Kelton, N. LaMonica, S. Malik, N. Marder, L. Mielke, and T. Siegel. We also thank W. Maas for helpful discussions; and we are grateful to B. Hamilton for typing the manuscript. This work was supported by NSF Grant PCM78-08906 and NIH Grants GM26863 and GM29085 to D.F.

REFERENCES

1. Barth, P.T. (1979) RP4 and R300B as wide host-range plasmid cloning vehicles. In Plasmids of Medical, Environmental and Commercial Importance, K.N. Timmis, and A. Puhler, Eds. Elsevier/North Holland, Amsterdam/New York, pp. 399-410.
2. Barth, P.T., K. Ellis, D.H. Bechhofer, and D.H. Figurski (1984) Involvement of kil and kor genes in the phenotype of a host-range mutant of RP4. Mol. Gen. Genet. (in press).
3. Barth, P.T., and N.J. Grinter (1974) Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bact. 120:618-630.
4. Bechhofer, D.H., and D.H. Figurski (1983) Map location and nucleotide sequence of korA, a key regulatory gene of promiscuous plasmid RK2. Nucl. Acids Res. 11:7453-7469.
5. Burkardt, H.J., G. Reiss, and A. Puhler (1979) Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68, and RK2 are identical. J. Gen. Microbiol. 114:341-348.
6. Cowan, P., and V. Krishnapillai (1982) Tn7 insertion mutations affecting the host range of promiscuous IncP-1 plasmid R18. Plasmid 8:164-174.
7. Datta, N., and R.W. Hedges (1972) Host ranges of R-factors. J. Gen. Microbiol. 70:453-460.

8. Figurski, D.H., and D.R. Helinski (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci., USA. 76:1648-1652.
9. Figurski, D.H., R.J. Meyer, and D.R. Helinski (1979) Suppression of ColE1 replication properties by the IncP-1 plasmid RK2 in hybrid plasmids constructed in vitro. J. Mol. Biol. 133:295-318.
0. Figurski, D.H., R.F. Pohlman, D.H. Bechhofer, A.S. Prince, and C.A. Kelton (1982) The broad host range plasmid RK2 encodes multiple kil genes potentially lethal to Escherichia coli host cells. Proc. Natl. Acad. Sci., USA 79:1935-1939.
1. Guiney, D.G., and E. Yakobson (1983) Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. Proc. Natl. Acad. Sci., USA 80:3595-3598.
2. Ingram, L., M.H. Richmond, and R.B. Sykes (1973) Molecular characterization of the R factors implicated in the carbenicillin resistance in a sequence of Pseudomonas aeruginosa strains isolated from burns. Antimicrob. Agents and Chemother 3:279-288.
3. Jacob, F., S. Brenner, and F. Cuzin (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329-348.
4. Lanka, E., R. Lurz, and J.P. Furste (1983) Molecular cloning and mapping of SphI restriction fragments of plasmid RP4. Plasmid 10:303-307.
5. Meyer, R., D. Figurski, and D.R. Helinski (1977) Phyiscal and genetic studies with restriction endonucleases on the broad host range plasmid RK2. Mol. Gen. Genet. 152:129-135.
6. Meyer, R., and D.R. Helinski (1977) Unidirectional replication of the P-group plasmid RK2. Biochem. Biophys. Acta 478:109-113.
7. Meyer, R., and M. Hinds (1982) Multiple mechanisms for expression of incompatibility by broad-host-range plasmid RK2. J. Bact. 152:1078-1090.
8. Olsen, R.H., and P. Shipley (1973) Host range and properties of the Pseudomonas aeruginosa R factor R1822. J. Bact. 113:772-780.
9. Pohlman, R.F., and D.H. Figurski (1983) Conditional lethal mutants of the kilB determinant of broad host range plasmid RK2. Plasmid 10:82-95.
0. Pohlman, R.F., and D.H. Figurski (1983) Essential genes of plasmid RK2 in Escherichia coli: trfB region controls a kil gene near trfA. J. Bact. 156:584-591.
1. Roberts, J.W. (1969) Termination factor for RNA synthesis. Nature, London 224:1168-1174.
2. Schmidhauser, T.J., M. Filutowicz, and D.R. Helinski (1983) Replication of derivatives of the broad host range plasmid RK2 in two distantly related bacteria. Plasmid 9:325-330.
3. Scott, J.R. (1984) Regulation of plasmid replication. Microbiol. Rev. 48:1-23.

24. Smith, C.A., and C.M. Thomas (1983) Deletion mapping of kil and kor functions in the trfA and trfB regions of broad host range plasmid RK2. Mol. Gen. Genet. 190:245-254.
25. Smith, C.A., and C.M. Thomas (1984) Nucleotide sequence of the trfA gene of broad host-range plasmid RK2. J. Mol. Biol. 175:251-262.
26. Stalker, D.M., C.M. Thomas, and D.R. Helinski (1981) Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:8-12.
27. Thomas, C.M. (1981) Complementation analysis of replication and maintenance of broad host range plasmids RK2 and RP1. Plasmid 5:277-291.
28. Thomas, C.M., A.A.K. Hussain, and C.A. Smith (1982) Maintenance of broad host range plasmid RK2 replicons in Pseudomonas aeruginosa. Nature 298:674-676.
29. Thomas, C.M., R. Meyer, and D.R. Helinksi (1980) Regions of broad host range plasmid RK2 which are essential for replication and maintenance. J. Bact. 141:213-222.
30. Thomas, C.M., D.M. Stalker, and D.R. Helinski (1981) Replication and incompatibility properties of segments of the origin region of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:1-7.
31. Villarroel, R., R.W. Hedges, R. Maenhaut, J. Leemans, G. Engler, M. Van Montagu, and J. Schell (1983) Heteroduplex analysis of P-plasmid evolution: The role of insertion and deletion of transposable elements. Mol. Gen. Genet. 189:390-399.
32. Young, C., D.H. Bechhofer, and D.H. Figurski (1984) Gene regulation in plasmid RK2: Positive control by korA in the expression of korC. J. Bact. 157:247-252.

REPLICATION DETERMINANTS OF THE BROAD HOST-RANGE PLASMID RSF1010

Peter Scholz, Volker Haring, Eberhard Scherzinger,
Rudi Lurz, Miroslawa M. Bagdasarian,* Heinz Schuster,
and Michael Bagdasarian*

Max-Planck-Institute of Molecular Genetics
Abteilung Schuster
1000 Berlin 33
Ihnestrasse 63-73, Germany

INTRODUCTION

RSF1010 is a small (8.7 kb), multicopy plasmid of the incompatibility group IncQ that confers resistances to streptomycin and sulfonamide to its host cells (1). It is very similar or identical with the 2 other representatives of the IncQ group, R300 (2) and R1162 (3). One of the most striking properties of this plasmid is its extraordinary broad host-range among gram-negative bacteria. This feature has made it attractive as a replicon for construction of vectors for gene cloning outside of Escherichia coli species. Both general- and special-purpose cloning vectors, derived from RSF1010, have proved very useful for gene manipulation in different species of soil bacteria (for review, see Ref. 4,5).

The molecular regulatory mechanisms that govern host-range specificity of plasmids are not well understood. This fact has prompted studies on the mechanism of replication, copy-number control, and maintenance of RSF1010 as a model plasmid that offers the convenience of small size and well-known physical structure (6,7). The picture emerging from these studies is that of a replicon encoding multiple essential replication genes that render the plasmid replication independent of several vital replication functions of the host cell.

*Unit for Applied Cell and Molecular Biology, Umea University, S-901 87 Umea, Sweden.

ESSENTIAL REGIONS OF RSF1010

The origin of vegetative replication (oriV) has been located at the coordinate of 2.6 kb on the physical map of RSF1010 (Fig. 1 and 2) by electron microscopic examination of replicating molecules linearized at the unique EcoRI-sensitive site of the plasmid (8). These measurements were performed on the replicative intermediates isolated from E. coli cells. They could not therefore provide adequate information on the mechanism of RSF1010 replication in other bacterial species. To answer the question whether in different host bacteria RSF1010 replication could be initiated specifically from other origins than in E. coli, we have examined, by electron microscopy, replicative intermediates of this plasmid isolated from Pseudomonas aeruginosa. Our measurements have shown (Fig. 1) that the replication of RSF1010 in this bacterium starts preferentially at the coordinate 2.65 kb. This location of oriV has been confirmed by measurements performed on replicating molecules linearized at the PvuII-sensitive sites (results not shown). From these results it is clear that in 2 organisms as different as E. coli and P. aeruginosa the same region of the plasmid, and therefore most probably the same origin, is used to initiate the replication of RSF1010. If this is indeed the case, other plasmid-coded replication or maintenance determinants must be responsible for the broad host-range specificity of this plasmid.

Restriction endonuclease cleavage analysis of RSF1010 indicated that the region comprising oriV should be contained within the stretch of the molecule spanned by the fragments HaeII (1.2-2.3 kb), HaeII (2.3-2.4 kb) and HaeII (2.4-3.3). However, attempts to generate self-replicating mini-plasmids from these 3 HaeII fragments ligated to different DNA fragments carrying antibiotic resistance genes were unsuccessful. This suggested the possibility that genes essential for replication or maintenance of RSF1010 are located in regions of the plasmid distant from oriV. To identify these regions, we have constructed hybrid plasmids carrying different DNA fragments of RSF1010 cloned into an unrelated plasmid vector and determined their ability to support the replication of the oriV-carrying HaeII fragments. One such helper plasmid was constructed by inserting a 5.0-kb SstI fragment from pKT228 (RSF1010 carrying Tn3 insertion at coordinate 4.2 kb) into the single SstI site of the vector pKT101 (7). The resulting recombinant plasmid, pMMB2, contains RSF1010 sequences located between coordinates 4.2 and 8.7 kb as well as a 0.3-kb segment of Tn3 (Fig. 2). E. coli strains carrying pMMB2 were used subsequently as recipients for recombinant DNA obtained by ligation of HaeII-digested RSF1010 DNA in the presence of nonself-replicating HaeII fragments carrying ampicillin- or chloramphenicol-resistance genes derived from a temperature-sensitive plasmid pHSG415 (9). Ap^r and Cm^r clones were selected and analyzed for the presence of satellite plasmids. All were found to contain, in addition to pMMB2, small plasmids. The smallest Ap^r plasmid, pMMB4 (4.5 kb), contained HaeII fragments [(1.2-2.3 kb), (2.3-2.4kb), and (2.4-3.3 kb)] of RSF1010 and the HaeII Ap^r fragment of pHSG415 (Fig. 2). The smallest one of the Cm^r plasmids, pMMB12, contained the same HaeII fragments of RSF1010 ligated to the HaeII Cm^r fragment derived from pHSG415 (Fig. 2). The structure of the later miniplasmid was confirmed by electron microscopic analysis of heteroduplex molecules formed with RSF1010 (7).

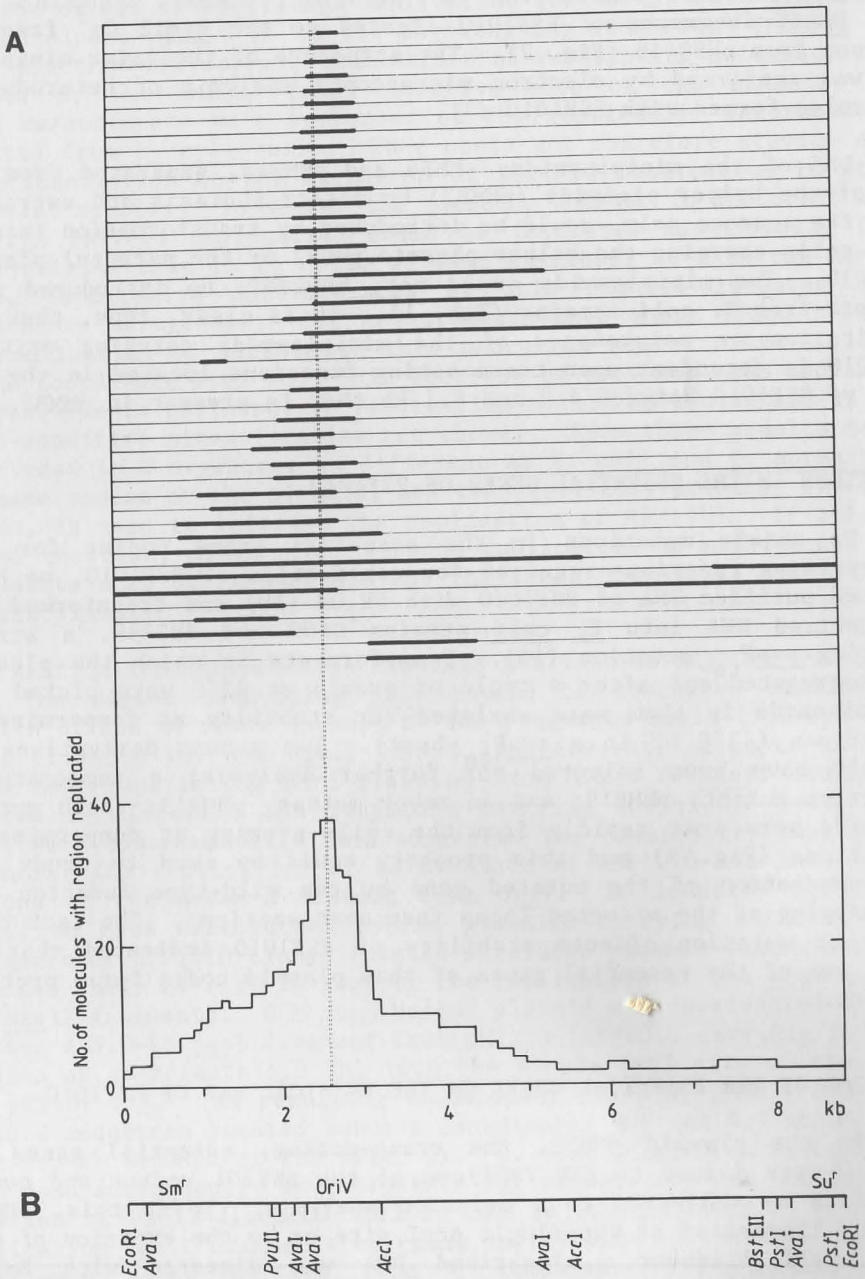
DNA of the miniplasmids, pMMB4 and pMMB12, separated from the DNA of the helper plasmids (pMMB2) by electrophoresis and extracted from the agarose gels, could be introduced by transformation into E. coli cells carrying the helper plasmid pMMB2 or the parental plasmid RSF1010. The miniplasmids could not, however, be introduced into plasmid-free E. coli strains (Tab. 1). It is clear, thus, that the replication or maintenance of the miniplasmids carrying oriV of RSF1010 is dependent upon trans-acting functions located in the region of RSF1010 between 4.2 and 8.7 kb that is present in pMMB2.

MUTATIONS IN THE ESSENTIAL GENES OF RSF1010

To obtain mutations in the essential genes coding for the trans-acting functions required for replication of RSF1010, we have treated purified DNA of RSF1010 with NH_2OH (10) and transformed the mutagenized DNA into E. coli strains C600 and RH2358, a strain carrying $supF_{ts}$ mutation (11). Transformants in which the plasmid was segregated out after a cycle of growth at 42°C were picked and the plasmids in them were analyzed for stability at nonpermissive conditions (42°C or in a $supF_{ts}$ host). Two mutant derivatives of RSF1010 have been selected for further analysis; a temperature-sensitive mutant, pMMB19; and an amber mutant, pMMB13. Both mutant plasmids were lost rapidly from the cells growing at nonpermissive conditions (Fig. 3) and this property could be used to study the complementation of the mutated gene by the wild-type function and for mapping of the affected locus (see next section). The fact that an amber mutation affects stability of RSF1010 indicated that at least one of the essential genes of this plasmid codes for a protein product.

LOCATION OF THE ESSENTIAL GENES ON THE PHYSICAL MAP OF RSF1010

In the plasmid pMMB2, the trans-acting, essential genes of RSF1010 were joined to the replicon of the pKT101 vector and could therefore be subjected to a deletion analysis. To do this, pMMB2 DNA was linearized at the single AccI site or by the excision of the small PstI fragment. Linearized DNA was digested with Bal31 nuclease, religated, and used to transform strain C600 to Km^r. Two series of deletions were thus obtained, one originating at the AccI site within RSF1010 sequences and one entering the RSF1010 segment



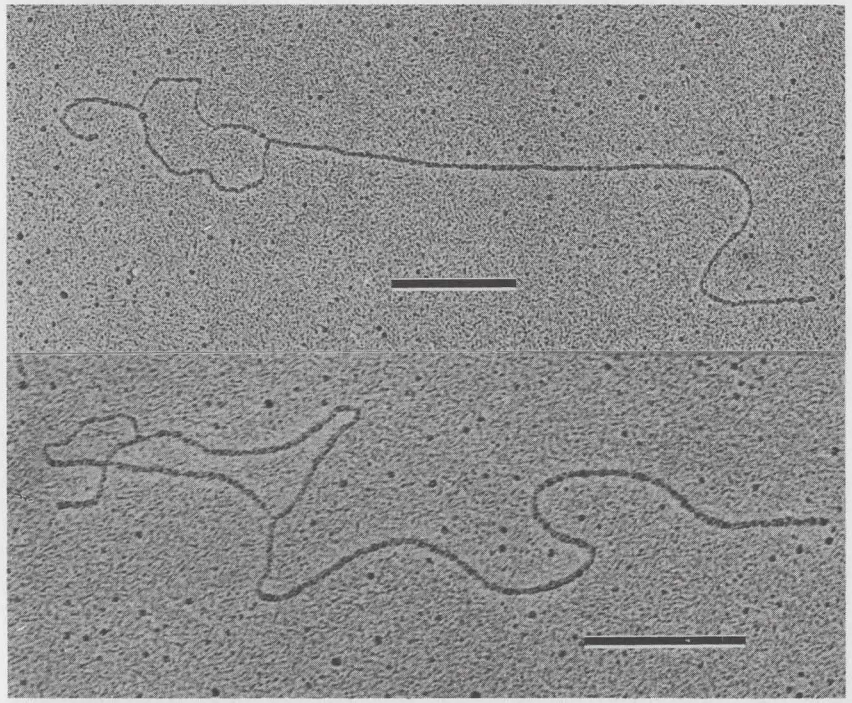


Fig. 1. Partially replicated molecules of RSF1010 isolated from Pseudomonas aeruginosa cleaved with EcoRI and visualized by electron miscroscopy. Upper part: Line drawings and histogram of RSF1010 replicative intermediates. Middle part: Restriction endonuclease cleavage map of RSF1010. Lower part: Electron micrographs of replicating molecules of RSF1010 from P. aeruginosa, linearized with EcoRI. The bar represents 1 kb.

from the PstI site (Fig. 4). A third series of deletions, entering the RSF1010 segment from the end opposite to the PstI end was obtained from DNA linearized with SstI. In this case, the parental plasmid used was a previously constructed derivative of pMMB2, Δ38, in which about 1 kb around the single EcoRI site had been deleted to remove the adjacent SstI site (Fig. 4). The end point of the deletions, mapped by electron microscopic analysis of heteroduplex molecules formed with parental plasmids, are shown in Fig. 4. All deletion derivatives of pMMB2 obtained this way were tested for their ability to provide the functions required for the replication and maintenance of the miniplasmid, pMMB12, and for the rescue of RSF1010 mutants pMMB19 and pMMB13. The results indicated that functions essential for the replication of the oriV miniplasmid are located on both sides of the single AccI site present in the plasmid pMMB2 (Fig. 4). Mutant rescue experiments have shown that pMMB2 de-

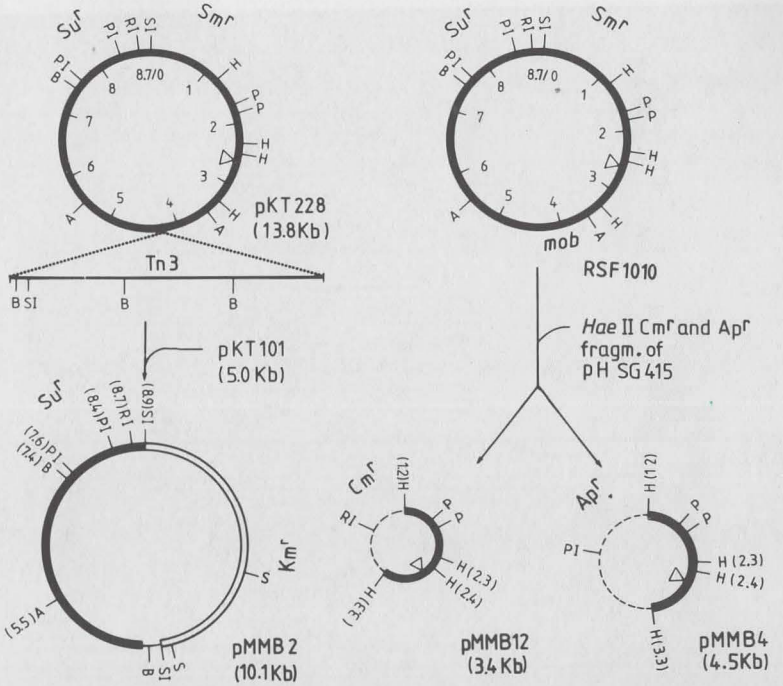


Fig. 2. Construction of plasmids carrying replication determinants of RSF1010: oriV and rep. The thick solid line represents sequences derived from RSF1010. Restriction endonuclease cleavage sites (Ref. 6, 7) are indicated by the code: A, AccI; B, BstEII; RI, EcoRI; H, HaeII; P, PvuII; PI, PstI; S, SmaI; SI, SstI. Of the numerous HaeII sites in RSF1010, only those present in the oriV region are indicated. The numbers in parenthesis refer to coordinates in parental RSF1010 molecule (in kb pairs from the unique SstI site). The open triangle indicates the origin of RSF1010 replication (8).

rivatives in which deletions entered the RSF1010 segment from the PstI site could rescue the mutation in pMMB19 but not that in pMMB13, while derivatives with deletions around the AccI site could rescue the mutation in pMMB13 but not in pMMB19. Moreover, deletions Δ63 and Δ67 entering the RSF1010 segment from the end opposite to the PstI end and unable to complement for the replication of the oriV miniplasmids were nevertheless able to rescue mutations in both pMMB19 and pMMB13 (Fig. 4). The region of RSF1010 comprised between the RSF1010 coordinates 4.2 - 8.7 may thus be subdivided into 3 segments, each encoding a function essential for replication or maintenance of plasmids with RSF1010 origin. We have proposed to call these essential genes repA, repB, and repC. These results have been confirmed by the demonstration that an in vitro DNA replication sys-

Tab. 1. Transformation frequency of miniplasmids carrying the origin of RSF1010 in E. coli.

Plasmid		
Resident	Incoming	Transformants / 1 µg DNA
none	pMMB4	< 200
none	pMMB12	< 200
pKT101	pMMB4	< 200
pKT101	pMMB12	< 200
pMMB2	pMMB4	6.0×10^5
pMMB2	pMMB12	8.0×10^5
RSF1010	pMMB4	1.8×10^5

Approximately 10^9 competent cells of E.coli C600 were transformed with 5 ng of mini-plasmid DNA purified by agarose gel electrophoresis. Transformants were selected for Ap^r in the case of pMMB4 and for Cm^r in the case of pMMB12.

tem requires the addition of protein products of all 3 rep genes, encoded on the plasmid pMMB2, in order to replicate RSF1010 DNA. The products of genes repA, repB, and repC have been purified to homogeneity by using an in vitro complementation test as assay for their activity. Finally the positions of the individual rep genes, shown in Fig. 4, have been confirmed by complementation tests run with extracts, prepared from strains carrying deletion derivatives of pMMB2, to which purified rep proteins were added in the in vitro DNA replication assay (7).

NUCLEOTIDE SEQUENCE OF repA GENE

Cloning experiments have shown that the 1.24-kb BalI fragment located between AccI and PstI sites of RSF1010 (Fig. 4 and 5) contains a complete functional repA gene. Moreover, repA protein purified from cells carrying this 1.2-kb BalI fragment, isolated from the thermosensitive plasmid pMMB19 and cloned into an expression vector, was temperature-sensitive in an in vitro assay (unpub. data). These determinations provided direct evidence for the location of the structural gene for the repA protein (Fig. 4 and 5). They showed also that repA gene is transcribed in the direction from AccI towards the BssHII and BstEII site.

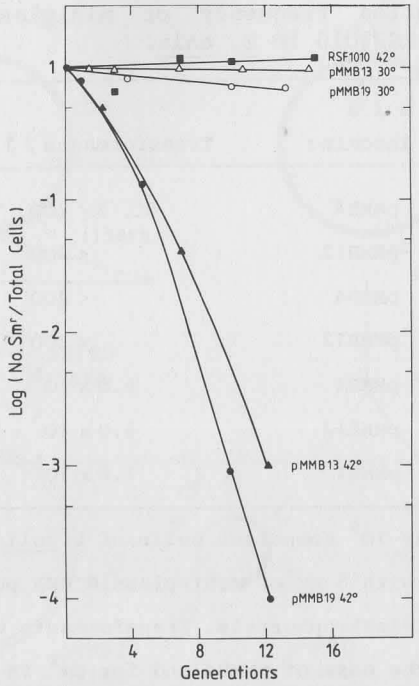


Fig. 3. Stability of RSF1010 derivatives at different temperatures. Logarithmically growing cultures of E. coli C600 carrying RSF1010 or pMMB19 and E. coli RH2358 [sup Fts (11)] carrying pMMB13 were transferred to the indicated temperature. At time intervals, suitable dilutions of the cultures were plated on Luria Broth agar with or without streptomycin. Colonies were scored after 24 hr at 30°C.

To carry on a more detailed analysis of the region containing the repA gene and to reveal possible signals for its regulation, we have determined the complete nucleotide sequence of the DNA fragment comprised between the AccI site at coordinate 5.5 kb and BalI site at coordinate 6.9 kb. The nonrandom sequencing strategy of Poncz et al. (12) was used to generate overlapping fragments that were sequenced by the dideoxy-chain termination method (13).

The sequence thus obtained contains several open frames reading in the direction from AccI towards BssHII site. The correct reading frame for the repA gene has been selected by taking into account the subunit size of the purified repA protein and of the sequence of 53 amino acids from its $-NH_2$ terminus (Scholz, Scherzinger, Haring, Wittmann, and Bagdasarian, to be published elsewhere).

The nucleotide sequence of the 1243-bp BalI fragment of RSF1010, containing the entire repA gene, is shown in Fig. 5. One

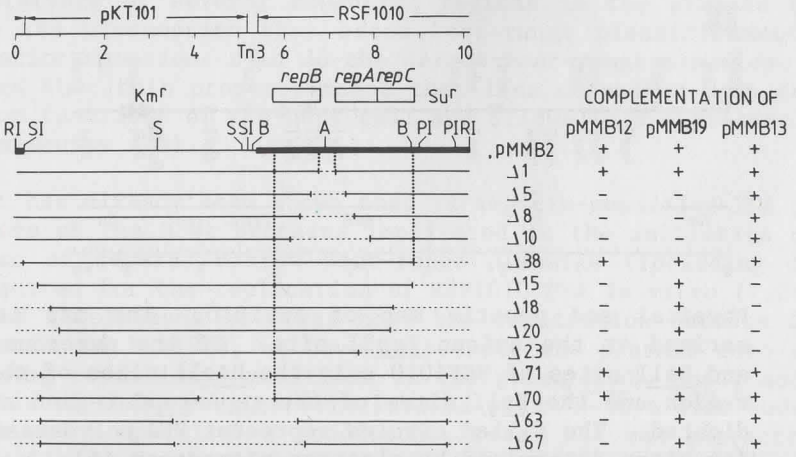


Fig. 4. Upper part: Physical and genetic map of pMMB2. Distances are given in kb pairs from the unique EcoRI site. Abbreviations for restriction endonucleases are explained in legend to Fig. 2. The thick solid line denotes sequences derived from RSF1010. Open boxes represent the positions of rep genes deduced from the data in this table, reference (7), and nucleotide sequence analysis.

Lower part: Diagrams of pMMB2 deletion derivatives used in this work. Broken lines represent the areas deleted, as determined by DNA-DNA heteroduplex analysis (7).

Table: Complementation of the replication ability of mutant RSF1010 derivatives by deletions of pMMB2. Strains containing the pairs of plasmids indicated were plated and grown at nonpermissive conditions without antibiotics. Individual colonies were then tested for the appropriate resistance markers. Positive complementation (+) was assigned to those pairs where at least 90% retained the marker of the RSF1010 derivative. Lack of complementation (-) indicates that 95% of the colonies have lost the marker of the RSF1010 derivative.

strand of DNA is displayed and the entire sequence is numbered, in both directions, beginning at the translational start codon, ATG, of the repA protein. The coding region of the gene is preceded, at positions -7 through -13, by a sequence -AAGGAGG- which is complementary to the end of the 16S ribosomal RNA and represents the ribosome binding site (14). We assume that the sequences -GTGACC-, at positions -49 through -44, and -CCACCT-, at positions -26 through -21, represent the RNA polymerase binding site (-35 region) (15) and the Pribnow box (-10 region) (15) of the repA gene promoter. This assumption, however, is based solely upon the location of these se-

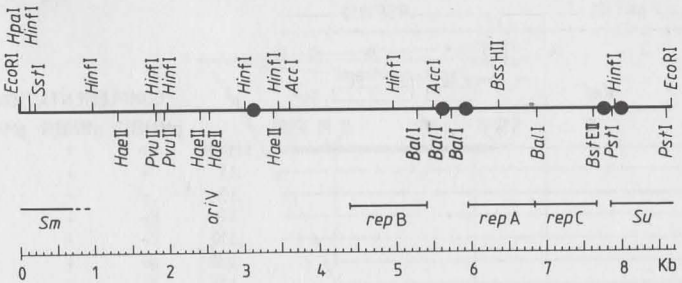


Fig. 5. Physical and genetic map of RSF1010. The map is linearized at the unique EcoRI site. Of the numerous HaeII and BalI sites of RSF1010 only the HaeII sites of the oriV region and the BalI sites of the repA-repC region are indicated. The filled circles represent RNA polymerase binding sites determined by electron microscopy (6).

quences close to and upstream of the Shine-Dalgarno sequence and the translational start codon of the gene.

The coding sequence of the repA gene contains 837 bp and terminates with GCC (Ala) followed by a TAA stop codon. Since the initiator Met residue is not present in the mature repA protein this sequence codes for 278 amino acids. The molecular weight of the polypeptide coded for by this sequence is 29.7 kd-- a value that is in good agreement with the subunit size of 29.0 kd determined for the purified repA protein by the SDS-polyacrylamide electrophoresis (unpub. results).

There is no recognizable transcription termination structure (15) following the coding region of the gene either in the sequence presented in Fig. 5 or downstream of it up to the region that represents coding sequences of the repC gene. We must assume, therefore, that the genes repA and repC are transcribed as a unit and, therefore, most probably, share at least some regulatory features.

REQUIREMENTS OF HOST FUNCTIONS FOR THE REPLICATION OF RSF1010

As shown in this and previous work (7), RSF1010 requires 3 proteins encoded by its own genome to start the replication from its origin. Both the number of essential genes and their arrangement on the physical map of this plasmid distinguishes it from the narrow host-range plasmids studied to date, such as ColE1, R1, R6-5, R6K, F, and pSC101, which have their replication determinants clustered around the origin of replication (16-22). The genetic organization of RSF1010 resembles rather that of another broad host-range plasmid, RK2/RP1/RP4, in which several essential replication and maintenance genes are scattered over a large portion of the genome (23).

The existence of several essential regions in the plasmid RK2 has led to the presumption that broad host-range plasmids encode more replication functions than do the narrow host-range plasmids. It is believed that this property makes them less dependent upon the replication functions of the host cell and allows thus a greater degree of promiscuity (23).

It has already been shown that rifampicin-sensitive RNA polymerase, one of the host proteins implicated in the initiation of replication of several narrow host-range plasmids (including dv), is not required for the replication of RSF1010 DNA in vitro (7,24). To test the requirement of other host DNA replication factors for the replication of RSF1010, we have introduced the plasmid into strains containing conditional mutations in the genes dnaB, dnaC, and dnaG. The cells of these strains were labeled with ^{3}H-thymidine under permissive and nonpermissive conditions, total DNA was extracted from these cells by lysis with Sarkosyl, and analyzed by CsCl-ethidium bromide gradient centrifugation.

Under permissive conditions (30°C) total radioactivity incorporated into the supercoiled DNA fraction, which represents plasmid DNA, amounts to 0.02 - 0.06 of the total radioactivity incorporated into the linear DNA fraction, composed predominantly of chromosomal DNA (Fig. 7, Tab. 2). This ratio corresponds to 9-28 copies of the plasmid per chromosome equivalent if the value of 3,750 kbp is taken as the size of the E. coli chromosome. These figures are in good agreement with the previous estimates of 10-50 copies of RSF1010 per cell (1). Under nonpermissive conditions (41°C) the incorporation of thymidine into the chromosomal DNA is drastically reduced whereas the incorporation into the supercoiled DNA fraction is reduced to a small extent (Fig. 7). In all 3 mutants tested there was considerably more thymidine incorporated into the plasmid DNA fraction than into the chromosome (Tab. 2). We conclude, therefore, that under conditions where either dnaB, dnaC, or dnaG function is inhibited, the replication of RSF1010 DNA continues. In a strain containing dnaZ mutation, RSF1010 seems to be present at such low copy number that its estimation by labeling and dye-buoyant density centrifugation of total cellular DNA was difficult even at permissive conditions. We consider this as an indication that DNA polymerase III, or at least some of its components, are required for the replication of RSF1010 in E. coli cells.

CONCLUDING REMARKS

Among plasmids that are able to replicate in different, distantly related, bacterial hosts the genetics of replication determinants has been investigated in only 2 cases, that of RP4/RK2 plasmid (23,25-27) and of RSF1010 (Ref. 7, this work). In both plasmids it was found that essential genes are scattered over a large portion

```
 BalI -190       -180       -170       -160       -150       -140
TGGCCAGCCCTGACGCTGTACGCCAAGCGCGATATGCCGAGCGCATGAAGGCCAAAGGGA
      -130       -120       -110       -100        -90        -80
TGCGTCAGCGCAAGTTCTGGCTGACCGACGACGAATACGAGGCGCTGCGCGAGTGCCTGG
       -70        -60        -50        -40        -30        -20
AAGAACTCAGAGCGGCGCAGGGCGGGGGTAGTGACCCCGCCAGCGCCTAACCACCAACTG
      -10          0         10         20         30
CCTGCAAAGGAGGCAATCA ATG GCT ACC CAT AAG CCT ATC AAT ATT CTG
                    Ala Thr His Lys Pro Ile Asn Ile Leu
            40         50         60         70
GAG GCG TTC GCA GCA GCG CCG CCA CCG CTG GAC TAC GTT TTG CCC
Glu Ala Phe Ala Ala Ala Pro Pro Pro Leu Asp Tyr Val Leu Pro
    80         90        100        110        120
AAC ATG GTG GCC GGT ACG GTC GGG GCG CTG GTG TCG CCC GGT GGT
Asn Met Val Ala Gly Thr Val Gly Ala Leu Val Ser Pro Gly Gly
          130        140        150        160
GCC GGT AAA TCC ATG CTG GCC CTG CAA CTG GCC GCA CAG ATT GCA
Ala Gly Lys Ser Met Leu Ala Leu Gln Leu Ala Ala Gln Ile Ala
   170        180        190        200        210
GGC GGG CCG GAT CTG CTG GAG GTG GGC GAA CTG CCC ACC GGC CCG
Gly Gly Pro Asp Leu Leu Glu Val Gly Glu Leu Pro Thr Gly Pro
          220        230        240        250
GTG ATC TAC CTG CCC GCC GAA GAC CCG CCC ACC GGC ATT CAT CAC
Val Ile Tyr Leu Pro Ala Glu Asp Pro Pro Thr Ala Ile His His
   260        270        280        290        300
CGC CTG CAC GCC CTT GGG GCG CAC CTC AGC GCC GAG GAA CGG CAA
Arg Leu His Ala Leu Gly Ala His Leu Ser Ala Glu Glu Arg Gln
          310        320        330        340
GCC GTG GCT GAC GGC CTG CTG ATC CAG CCG CTG ATC GGC AGC CTG
Ala Val Ala Asp Gly Leu Leu Ile Gln Pro Leu Ile Gly Ser Leu
   350        360        370        380     BssHII
CCC AAC ATC ATG GCC CCG GAG TGG TTC GAC GGC CTC AAG CGC GCC
Pro Asn Ile Met Ala Pro Glu Trp Phe Asp Gly Leu Lys Arg Ala
          400        410        420        430
GCC GAG GGC CGC CGC CTG ATG GTG CTG GAC ACG CTG CGC CGG TTC
Ala Glu Gly Arg Arg Leu Met Val Leu Asp Thr Leu Arg Arg Phe
```



```
    440        450        460        470        480
CAC ATC GAG GAA GAA AAC GCC AGC GGC CCC ATG GCC CAG GTC ATC
His Ile Glu Glu Glu Asn Ala Ser Gly Pro Met Ala Gln Val Ile
          490        500        510        520
GGT CGC ATG GAG GCC ATC GCC GCC GAT ACC GGG TGC TCT ATC GTG
Gly Arg Met Glu Ala Ile Ala Ala Asp Thr Gly Cys Ser Ile Val
    530        540        550        560        570
TTC CTG CAC CAT GCC AGC AAG GGC GCG GCC ATG ATG GGC GCA GGC
Phe Leu His His Ala Ser Lys Gly Ala Ala Met Met Gly Ala Gly
          580        590        600        610
GAC CAG CAG CAG GCC AGC CGG GGC AGC TCG GTA CTG GTC GAT AAC
Asp Gln Gln Gln Ala Ser Arg Gly Ser Ser Val Leu Val Asp Asn
    620        630        640        650        660
ATC CGC TGG CAG TCC TAC CTG TCG AGC ATG ACC AGC GCC GAG GCC
Ile Arg Trp Gln Ser Tyr Leu Ser Ser Met Thr Ser Ala Glu Ala
          670        680        690        700
GAG GAA TGG GGT GTG GAC GAC GAC CAG CGC CGG TTC TTC GTC CGC
Glu Glu Trp Gly Val Asp Asp Asp Gln Arg Arg Phe Phe Val Arg
    710        720        730        740        750
TTC GGT GTG AGC AAG GCC AAC TAT GGC GCA CCG TTC GCT GAT CGG
Phe Gly Val Ser Lys Ala Asn Tyr Gly Ala Pro Phe Ala Asp Arg
          760        770        780        790
TGG TTC AGG CGG CAT GAC GGC GGG GTG CTC AAG CCC GCC GTG CTG
Trp Phe Arg Arg His Asp Gly Gly Val Leu Lys Pro Ala Val Leu
    800        810        820        830        840
GAG AGG CAG CGC AAG AGC AAG GGG GTG CCC CGT GGT GAA GCC TAA
Glu Arg Gln Arg Lys Ser Lys Gly Val Pro Arg Gly Glu Ala ***
       850        860        870        880        890        900
GAACAAGCACAGCCTCAGCCACGTCCGGCACGACCCGGCGCACTGTCTGGCCCCCGGCCT
       910        920        930        940        950        960
GTTCCGTGCCCTCAAGCGGGGCGAGCGCAAGCGCAGCAAGCTGGACGTGACGTATGACTA
       970        980        990       1000       1010       1020
CGGCGACGGCAAGCGGATCGAGTATCAGCGGCCCGGAGCCGCTGGGCGCTGATGATCTGC
      1030        BalI
GCATCCTGCAAGGGCTGGTGGCCA
```

Fig. 6. Nucleotide sequence of the BalI fragment of RSF1010 containing the repA gene. The putative promoter sequences of the gene are enclosed in solid line boxes and the ribosome binding site in broken line box. Nucleotides are numbered, in both directions, from the translation start codon, ATG of the repA coding sequence. Amino acids of repA protein are shown under their respective codon triplets. The stop codon of repA coding sequence is indicated by asterisks.

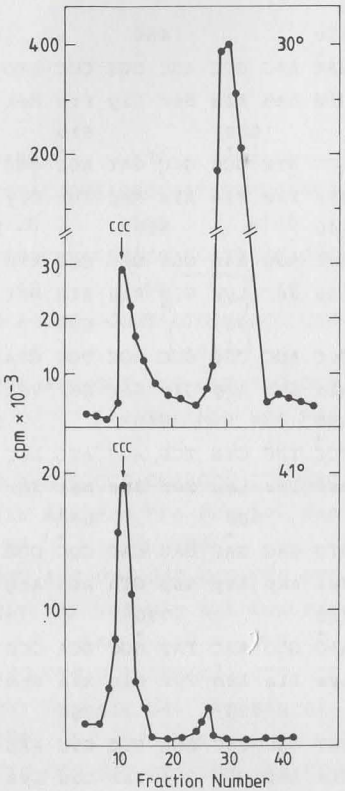


Fig. 7. Dye-buoyant density centrifugation profile of total DNA of a dnaG [RSF1010] strain labeled at permissive (30°C) and nonpermissive (41°C) temperature. One ml culture (5×10^8 cells) of the strain BW86 [RSF1010] (dnaG3 deoB thyA) was placed at temperatures indicated and labeled by the addition of ^{3}H-thymidine (0.04 mCi, 3.5 Ci per 1 mmol) for 60 min. The cultures were lysed with Sarkosyl (1.0%) and centrifuged to equilibrium in CsCl-Ethidium bromide density gradient. Fractions of the gradient were collected on Whatman GF/C discs, washed with 5% trichloroacetic acid, then ethanol, dried, and counted in a liquid scintillation counter.

of the genome. Moreover, the number of essential genes in broad host-range plasmids is greater than in the plasmids with narrow host specificity. Of the 2 broad host-range plasmids studied so far, RSF1010 represents a more dramatic example of this type of genetic structure. Despite the relatively small size, about 50% of its genome is taken up by essential genes.

The rep genes of RSF1010, described in this and previous papers (7), code for proteins that are specifically required for the repli-

Tab. 2. Incorporation of thymidine into RSF1010 DNA in dna mutants.

Chromosomal mutation	cpm ccc.DNA / linear DNA	
	30°	42°
dnaB	0.020	5.00
dnaG	0.065	12.02
dnaC	0.170	3.20

Cultures were labelled and analysed as described in the legend to Fig. 7. The counts in the peaks were summed to determine the ratios given in the table. Strains were dnaB165/70 thyA thi [RSF1010], dnaC2 thyA dra [RSF1010] and dnaG3 deoB thyA [RSF1010].

cation of plasmids carrying the replication origin of RSF1010 in vivo and in vitro. It could be expected, therefore, that their role would be either to replace the functions of some host cell DNA replication proteins in the replication of RSF1010 or to serve as a functional (adaptational) link between the replication factors of the host and the initiation factor of the plasmid. On the basis of the in vivo experiments reported in this work it cannot be ruled out that the replication factors of RSF1010 act by stabilizing the temperature-sensitive dnaB, dnaC, and dnaG proteins and thus mimic the independence of RSF1010 replication of these host proteins. However, parallel experiments performed with the in vitro replication system indicated that, at least in E. coli, the 3 dna proteins of the host are replaced by the rep proteins of RSF1010. The 3 rep proteins are specific for the RSF1010 template; they are not able to substitute for the dna functions of the host either in the chromosome replication in vivo or in the replication of ColE1 DNA in an in vitro system (unpub. data).

Differences in properties of the dnaB, dnaC, and dnaG proteins among gram-negative bacteria may constitute a barrier for those plasmids that rely on the functions of these host proteins for their replication. The example of RSF1010 shows that at least some of the broad host-range plasmids have evolved by acquiring their own genes that perform functions analogous to the dnaB, dnaC, and dnaG genes of E. coli but are specific for the replication of plasmid DNA.

ACKNOWLEDGMENTS

This work was supported in part by the grant No. GI-3-060-D from the Biomolecular Engineering Program of the Commission of European Communities.

REFERENCES

1. Guerry, P., J. van Embden, and S. Falkow (1974) Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bact. 117:619-630.
2. Barth, P., and N.J. Grinter (1974) Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bact. 120:618-630.
3. Meyer, R., M. Hinds, and M. Brasch (1982) Properties of R1162, a broad host range, high copy number plasmid. J. Bact. 150:552-562.
4. Bagdasarian, M., and K.N. Timmis (1982) Host:vector systems for gene cloning in Pseudomonas. Curr. Topics Microb. Immun. 96:47-67.
5. Lehrbach, P.R., and K.N. Timmis (1983) Genetic analysis and manipulation of catabolic pathways in Pseudomonas. Biochem. Soc. Symp. 48:191-219.
6. Bagdasarian, M., R. Lurz, B. Rückert, F.C.H. Franklin, M.M. Bagdasarian, J. Frey, and K.N. Timmis (1981) Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and host-vector system for gene cloning in Pseudomonas. Gene 16:237-247.
7. Scherzinger, E., M.M. Bagdasarian, P. Scholz, R. Lurz, B. Rückert, and M. Bagdasarian (1984) Replication of the broad host range plasmid RSF1010: Requirement of three plasmid-encoded proteins. Proc. Natl. Acad. Sci., USA 81:654-658.
8. De Graaff, J., J.H. Crosa, F. Heffron, and S. Falkow (1978) Replication of the non-conjugative plasmid RSF1010 in Escherichia coli K12. J. Bact. 134:1117-1122.
9. Hashimoto-Gotoh, T., F.C.H. Franklin, A. Nordheim, and K.N. Timmis (1981) Specific-purpose plasmid cloning vectors. I. Low copy number, temperature-sensitive, mobilization-defective pSC101-derived containment vectors. Gene 16:227-235.
10. Humphreys, G.D., G.A. Willshaw, H.R. Smith, and E.S. Anderson (1976) Mutagenesis of plasmid DNA with hydroxylamine: Isolation of mutants of multi-copy plasmids. Mol. Gen. Genet. 145:101-108.
11. Delcuve, G., T. Cabezon, A. Ghysen, A. Herzog, and A. Bollen (1977) Amber mutations in Escherichia coli essential genes: Isolation of mutants affected in the ribosomes. Mol. Gen. Genet. 157:149-153.

12. Poncz, M., D. Solowiejczyk, M. Ballantine, E. Schwartz, and S. Surrey (1982) "Nonrandom" DNA sequence analysis in bacteriophage M13 by the dideoxy chain termination method. Proc. Natl. Acad. Sci., USA 79:4298-4302.
13. Messing, J. (1983) New M13 vectors for cloning. Meth. in Enzym. 101:20-77.
14. Shine, J., and L. Dalgarno (1975) Determinant of cistron specificity in bacterial ribosomes. Nature 254:34-38.
15. Rosenberg, M., and D. Court (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. Ann. Rev. Genet. 13:319-353.
16. Backman, K., M. Betlach, H.W. Boyer, and C. Yanofsky (1978) Genetic and physical studies on the replication of ColE1-type plasmids. Cold Spring Harbor Symp. Quant. Biol. 43:69-76.
17. Leight, J., and S. Molin (1981) Replication control functions of plasmid R1 act as inhibitors of expression of a gene required for replication. Mol. Gen. Genet. 184:56-61.
18. Timmis, K.N., H. Danbara, G. Brady, and R. Lurz (1981) Inheritance functions of group IncFII transmissible antibiotic resistance plasmids. Plasmid 5:53-75.
19. Stalker, D.M., R. Kolter, and D.R. Helinski (1982) Plasmid R6K DNA replication. I. Complete nucleotide sequence of an autonomously replicating segment. J. Mol. Biol. 161:33-43.
20. Komai, N., T. Nishizawa, Y. Hayakawa, T. Murotsu, and K. Matsubara (1982) Detection and mapping of six mini F-encoded proteins. Mol. Gen. Genet. 186:193-203.
21. Lindner, P., G. Churchward, and L. Caro (1983) Plasmid pSC101 replication mutants generated by insertion of the transposon Tn1000. J. Mol. Biol. 170:287-303.
22. Thomas, C.M., R. Meyer, and D.R. Helinski (1980) Regions of broad host range plasmid RK2 which are essential for replication and maintenance. J. Bact. 141:213-222.
23. Diaz, R., and W.L. Staudenbauer (1982) Replication of the broad host range plasmid RSF1010 in cell-free extracts of Escherichia coli and Pseudomonas aeruginosa. Nucl. Acid Res. 10:4687-4702.
24. Figurski, D., and D.R. Helinski (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci., USA 76:1648-1652.
25. Thomas, C.M. (1981) Molecular genetics of broad host range plasmid RK2. Plasmid 5:10-19.
26. Thomas, C.M. (1981) Complementation analysis of replication and maintenance functions of broad host range plasmids RK2 and RP1. Plasmid 5:277-291.

REGULATION OF REPLICATION AND MAINTENANCE FUNCTIONS OF BROAD HOST-RANGE PLASMID RK2

C. M. Thomas, C. A. Smith, V. Shingler, M. A. Cross, A. A. K. Hussain,* and M. Pinkney

Department of Genetics
University of Birmingham
P. O. Box 363
Birmingham B 15 2TT, England

ABSTRACT

Replication of broad host-range plasmid RK2 depends on a cis-acting vegetative replication origin $\underline{oriV}_{RK2}$ and the polypeptide product(s) of the trans-acting gene trfA as well as on host-specified products. The trfA gene is the second cistron in a polycistronic unit whose first cistron may be kilD, one of 4 known RK2-specified kil loci (kilA, B, C, and D) which are inhibitory for bacterial host or plasmid vector in the absence of kor functions which suppress in trans the effect of their respective kil genes. Transcription of the operon containing trfA is negatively regulated by the products of both the trfB locus (alias korD and korA) and korB. The loci, trfB and korB, are expressed from a single transcriptional unit which we propose to be negatively autoregulated by the products of both loci, although an additional, weaker and unregulated transcript may also express korB. While deletions in the $\underline{oriV}_{RK2}$ region have indicated the presence of copy number control elements adjacent to and possibly overlapping with the minimal $\underline{oriV}_{RK2}$ segment, the overriding control of copy number seems to reside in the trfB and korB loci which in conjunction appear to reduce expression of the trfA gene to levels limiting for replication. Coregulation of trfA with kil genes may indicate that kil genes play a role in plasmid maintenance other than replication.

* Present address: Max-Planck Institut für Molekulare Genetik, Abteilung Wittman, D-1000 Berlin 33 (Dahlem), Federal Republic of Germany.

INTRODUCTION

Escherichia coli IncP-1 plasmid RK2 (similar and probably identical to RP1, RP4, R68, and R18) is capable of conjugal transfer between and stable maintenance in most gram-negative bacterial species (for a general review of this plasmid, see Ref. 1). Interestingly, it is reported elsewhere in this volume (2) that RK2 cannot replicate in gram-negative anaerobes such as Bacteroides spp., the first well documented example of a limit on the host range of RK2. A physical and genetic map of RK2 is shown in Fig. 1 using the recently revised size of 60 kb (3). The locations of plasmid genes are given by reference to these kb coordinates clockwise from the unique EcoRI site. In addition to genes (Pn^r, Tc^r, and Km^r) conferring resistance to penicillin (carried within the transposon Tn1 which is present in RK2), tetracycline, and kanamycin, respectively, about one third of the genome is composed of blocks of genes involved in conjugal DNA transfer (tral, tra2, and tra3; Ref. 4, 5, 6; a DNA primase, pri, Ref. 7; and the transfer origin oriT Ref. 8, 9). The rest of the genome contains the essential replication functions as well as kil and kor genes whose possible significance in plasmid maintenance is discussed briefly at the end of this paper.

For replication RK2 encodes $oriV_{RK2}$ (12.3 to 13.0 kb) (10-13), the origin from which vegetative replication proceeds unidirectionally anticlockwise (14), and trfA (16.0 to 17.3 kb) which encodes a polypeptide(s) essential for $oriV_{RK2}$ function (10,11,15). Replicons whose essential components consist of just these 2 RK2-derived loci

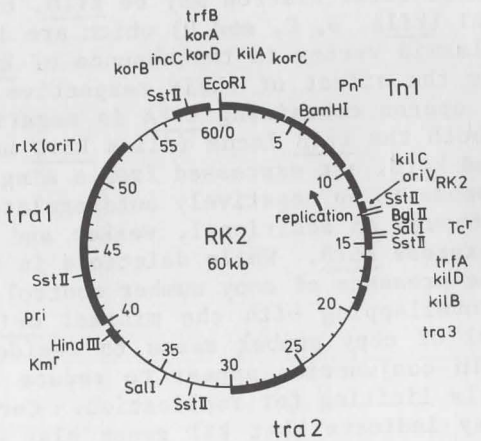


Fig. 1. Physical and genetic map of RK2. The loci shown are defined in the Introduction. The genome is calibrated in kb coordinates. The location of replication genes have been revised to the new 60 kb map by using EcoRI and SalI sites as reference points.

have at least part of the host range of parental RK2 (E. coli, Pseudomonas aeruginosa, P. putida, Azotobacter vinelandii, Acinetobacter calcoaceticus, Rhizobium meliloti, Agrobacterium tumefaciens, Rhodopseudomonas sphaeroides, Caulobacter crescentus; Ref. 16, 16a; this laboratory, unpub. data). Although only $oriV_{RK2}$ and trfA are essential for replication initial attempts to obtain small derivatives of RK2 by deletion analysis using partial digestion with the restriction endonuclease HaeII resulted in miniplasmids with DNA from 3 regions of the parental genome: 12.3 to 13.0 kb ($oriV_{RK2}$), 16.0 to 18.7 kb (trfA), and 57.6 to 59.6 kb (11). This latter region was designated the trfB region because it also appeared to provide a trans-acting function involved in plasmid maintenance (15). However, it now seems clear that at least part of the reason for the initial inability to delete this trfB region is that kilD lies adjacent to trfA (17,18), the 2 not being separable by HaeII digestion, so that kilD was present with trfA rendering it essential that korD (apparently the same locus as trfB and korA) be present in these minireplicons unless a mutation inactivates kilD. Since the the kilD locus appears only to interfere with maintenance of the plasmid carrying it, it may be different from the other RK2 kil loci previously described (10,19) which are host lethal in the absence of their equivalent kor (or kil-override) functions. However, this apparent difference may result from the way the locus was mapped.

In addition to the trfB/korA/korD locus, the trfB region present in these mini RK2 replicons also encodes a distinct locus, designated incC by us, which has been reported (19; K. Ellis and P. Barth, pers. comm.) to express in trans incompatibility towards RK2 and certain large derivatives of RK2 but not towards miniplasmids like those described above.

In this chapter, we describe studies carried out in E. coli which have mapped the genetic loci known to lie in or adjacent to the trfA and trfB regions of RK2. In addition, we have investigated the circuits involved in regulated expression of these genes and we show that regulation of trfA gene expression appears to be involved in plasmid copy number control.

RESULTS

Analysis of the trfA Region

Analysis of the trfA region has been facilitated by the use of a plasmid, pCT87 (15), which carries a mutant trfA region containing a point mutation rendering kilD inactive and which was obtained after deletion of the trfB (korD) region. The nature of this mutation is discussed below, but suffice it to say at this stage that the overall gene organization of this region and the nature of gene products is not altered by this single base change. Analysis of the

trfA region was carried out by transposon mutagenesis of pCT87 using Tn1723, a Km^r insertion derivative of Tn1722, the minor transposon of Tn1721 (20). Tn1723 has the useful property of having EcoRI sites 15 bp from each end of the transposon within its inverted repeats. This allows accurate mapping of the transposon's point of insertion as well as deletion of all but 30 bp of the whole transposon. Since Tn1723 appears to have a polar effect on transcriptional units into which it inserts, it can inactivate genes either by disrupting their coding sequences or simply by reducing their transcription. In the latter case, however, reactivation would be expected on deletion of the internal transposon EcoRI fragment while the remaining 35 bp insertion (30 bp of Tn1723 and a 5 bp of direct duplication of the target site) would be expected to give a frame shift in any coding sequence and so still to inactivate the coding sequence. Such analysis allowed us to define the limits of the region required for trfA activity and established that there is a substantial segment upstream of the trfA coding sequence where polar effects can abolish trfA expression (21). Using galK promoter probe vectors (pK01 and derivatives, Ref. 22) to determine the direction of transcription at each point of the transposon, insertion indicated that there is only anticlockwise transcription in the region required for trfA activity (21) (Fig. 2). The promoter from which this transcription originates has been more accurately mapped to coordinate 17.65 kb approximately, first, by RNA polymerase binding studies using retention of RNA polymerase-DNA fragment complexes on nitro-cellulose filters and DNase I protection studies (23), and second, by reverse transcriptase mapping of the 5'end of trfA mRNA produced in vivo (M. Pinkney, unpub. data). This mRNA has the same 5' ends in E. coli, Ps. aeruginosa and Ps. putida, indicating that RNA polymerases from all 3 species can recognize a common DNA sequence which is interesting, since, apparently, standard Pseudomonas spp. promoters are not efficiently used by E. coli RNA polymerase.

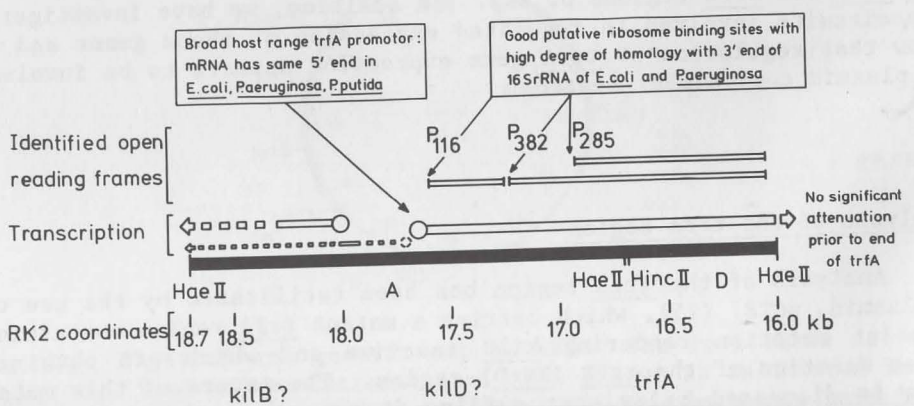


Fig. 2. Organization of the trfA region. See text for the experimental basis of these conclusions.

There is also clockwise transcription in the general trfA region as shown in Fig. 2. While weak clockwise transcription may originate from close to the trfA promoter, a second stronger transcription originates between coordinates 18.0 and 18.3 kb. As discussed below the promoter at this position may represent the transcriptional signal for the kilB gene.

Analysis of the gene products of the region required for trfA expression has been achieved by a combination of DNA sequence analysis (24) and identification of polypeptide products in maxicells (21). This region produces polypeptides of approximately 13 kd, 43 kd, and 32 kd, and since both the larger products are still produced by certain deletion derivatives which do not retain enough DNA to code for both separately, it seemed likely that their coding regions overlapped in some way. Analysis of polypeptide truncation products in maxicells containing internally deleted transposon insertion derivatives of pCT87 indicated that the 13 kd product must be the product of the region upstream from the trfA coding region itself in which transposon insertions have a polar effect on trfA activity. These analyses showed also that the coding region for the 43 dk polypeptide starts before that for the 32 kd polypeptide, and that a series of insertions which cause truncation of both the 32 kd and 43 kd polypeptides give products that consistently differ in size by 10-11 kd, suggesting that both polypeptides are translated from alternative translational starts within a single open reading frame. Only the 32 kd polypeptide is essential for trfA activity in E. coli but the 43 kd polypeptide may also have trfA activity.

This analysis of the organization of the trfA region is confirmed by DNA sequence analysis which reveals only one open reading frame (of 382 codons) large enough to code for either of the 32 or 43 kd polypeptides. This open reading frame is preceded by a good putative ribosome binding site and contains a second ribosome binding site prior to an internal ATG which would give a second translational product of 285 amino acids. Both the 382 and 285 amino acid polypeptides would be basic, consistent with a role as DNA binding proteins, and contain a sequence of amino acids which may be homologous to the family of α helix-turn-α helix DNA binding structures present in many dsDNA binding proteins (25).

Preceding the trfA open reading frame is another open reading frame of 116 codons whose location, with respect to transposon insertions, is consistent with its coding for the 13 kd polypeptide product observed in maxicells. For reasons discussed below we think that this may be the kilD gene product, so that, as summarized in Fig. 2 a polycistronic mRNA may be translated to give both a kil gene product and replication gene products. Interestingly the 13 kd polypeptide (P_{116}) is produced in much larger absolute amounts (rather than just by mole to mole ratio) than either the 43 kd (P_{382}) or 32 kd (P_{285}) polypeptides as estimated by both the amount

of coomassie blue stained material and the ^{14}C-amino acid label incorporation. Since quantitative estimates for the transcription indicate no significant attenuation prior to the end of the trfA gene (26), this must be due to either differential stability of mRNA segments, differential rates of translation initiation, and/or elongation or protein stability. The Shine/Dalgarno sequence is 9 base before the AUG for P_{116} but only 4 and 3 bases (far from ideal) for P_{382} and P_{285}, respectively suggesting that translational initiation efficiency may be at least one of the factors involved in this difference. The degree of efficiency with which P_{116} is expressed suggests that it may provide a useful set of signals for directing gene expression in a wide range of gram-negative bacterial species.

An additional locus previously proposed to lie within the trfA region is tra3, defined by a Tn7 insertion (in pRP101) which abolishes RP4 conjugal transfer and surface exclusion (6). While it has been demonstrated that tra3 is different from kilB (26a) from published data it is still possible that tra3 lies in the 16.0 to 18.7 kb region of RK2. However, accurate restriction mapping of pRP101 and subcloned segments in this laboratory indicates that the transposon insertion lies clockwise from the end of kilB and, therefore, completely outside the trfA region.

Analysis of the trfB Region

Analysis of the trfB region of RK2 and the adjacent segment containing korB has been carried out by a combination of insertion and deletion analysis, by the fusion of various segments of this region to galK promoter-probe plasmids and by analysis of polypeptide products in maxicells (17,27). The korA/korD/trfB locus within this region has been sequenced (28) and we have almost completed the DNA sequence of the rest of the 57.6 to 60.0 kb region. We have identified a single transcriptional unit running anticlockwise from a promoter which overlaps the HincII site at 59.3 kb (23) and continuing across all 3 known loci in this region trfB/korA/korD, incC, and korB (27). (The incC locus expresses incompatibility towards parental RK2-like plasmids (Ref. 27,29; K. Ellis and Barth, unpub. data; but its mode of action is unknown). All 3 loci are encoded clockwise of coordinate 56.8 kb. A second weaker transcriptional unit originating at an undefined point somewhere near coordinate 58.6 kb appears to transcribe across korB (27), Fig. 3.

The lengths of the polypeptides associated with incC activity (P_{38kD}) and korB activity (P_{49kD}) are sufficient to fully occupy the space between the promoter at 39.3 kb and the end of the korB gene at 56.8 kb, Fig. 3. Thus, if the trfB/korA/korD locus codes for a polypeptide, then it must either be a (processed?) subsegment of the incC-associated polypeptide or be translated from an overlapping reading frame in different phase from the incC open reading frame. The DNA sequence of the korA/trfB/korD region has

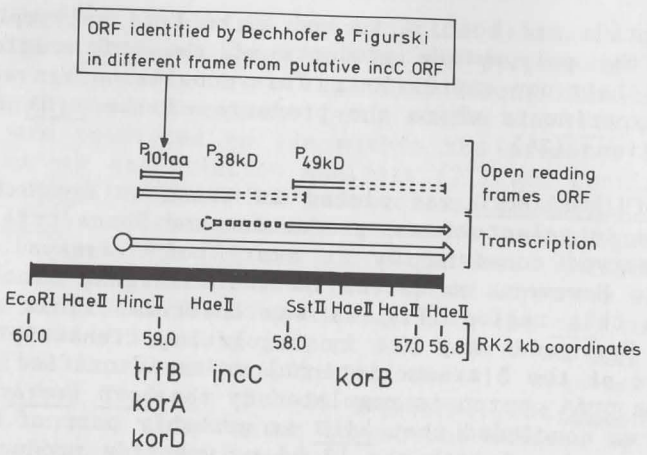


Fig. 3. Organization of the trfB, korB region. See text for the experimental basis of these conclusions.

been determined by Bechhofer and Figurski (28) and reveals both a 101 codon reading frame within the region defined as containing this locus and a second, out of phase reading frame starting just before this smaller open reading frame and continuing without termination across the end of the published sequence. We do indeed observe an approximately 14 kd polypeptide product which we proposed to be associated with trfB/korA/korD activity (27). Whether or not this corresponds to the product of the small reading frame in this region will only be resolved when we have point mutations which affect the product of only one of the two open reading frames. In view of the organization of transcription and translation signals in this region it seems highly unlikely that the trfB/korA/korD product could be a trans-acting RNA. The organization of the trfB region is summarized in Fig. 3.

Regulation of Genes in the trfA and the trfB Regions

In order to determine whether or not transcription in the trfA region is regulated by any of the products of the trfA or trfB regions various pKO plasmid derivatives with the trfA promoter transcribing across galK were used alone or in combination with compatible plasmids carrying various segments of these 2 RK2 regions (26). In all these experiments the level of pKO-derivative plasmid DNA was screened by rapid DNA isolation and gel electrophoresis and it was determined that the observed effects could not be explained simply by variations in plasmid copy number. Quantitative analysis of galactokinase production by fusions in the trfA transcript is such that the translation would produce: (1) only galactokinase; (2) both galactokinase and the 13 kd polypeptide; (3) galactokinase,

13 kd polypeptide and both 32 kd and 43 kd trfA polypeptides, suggested that the polypeptide products of the trfA region did not autoregulate their own expression. This conclusion was strengthened with other experiments where the products of the trfA region were provided in trans (26).

When trfB/korA/korD was placed in trans an approximately 10-fold decrease in galactokinase production and hence trfA transcription was observed consistently at every point assayed within the trfA operon. However, no effect on the divergent clockwise transcription in this region (Fig. 2) was observed. This result suggested that kor genes may act by regulating transcription of kil genes. Since of the 2 transcriptional units identified in this region only the trfA operon is regulated by the korD (korA/trfB) locus on its own, we concluded that kilD is probably part of this operon and may be associated with the 13 kd polypeptide product. Consistent with transcription from this promoter being responsible for kilD expression we have found that the only difference in DNA sequence between the wild-type trfA region (cloned always along with the trfB/korA/korD locus) and the mutant trfA region in pCT87 (which was cloned in the absence of the trfB region and which is effectively kilD$^-$) is a point mutation in the trfA promoter which reduces its homology with the E. coli concensus promoter sequence and which appears to reduce its strength in transcriptional initiation.

Since the kilB locus could at least partly overlap with the trfA region (17) it seemed possible that the clockwise transcription in this region might be responsible for the expression of kilB. The effect of plasmids carrying trfB, incC and korB in trans on galactokinase production by the trfA region transcriptional fusions was therefore determined and it was found that indeed the major clockwise transcription was reduced 10- to 15-fold when korB was present. Surprisingly, however, transcription of the trfA operon itself was also further reduced when korB was present, the combination of trfB and korB reducing transcription 100-fold (26). Since the clockwise transcription is negatively regulated when korB is present, as we proposed, we have tentatively assigned the major promoter in this region to an operon involved in kilB expression. Thus we find that the kilB and trfA promoters are both qualitatively and quantitatively different in their patterns of regulation of gene expression.

While it is possible that regulation of transcription might occur by means of premature termination of transcription it seems more likely that regulation occurs by interference with initiation of transcription, most likely by repressor protein binding to operator sites. The DNA sequence of the trfA promoter (identified both by RNA polymerase binding studies and reverse transcriptase mapping of mRNA 5' end) was compared to that of the putative kilB promoter (identified as the only region of good homology to the E. coli concensus promoter sequence between 2 Tn1723 insertion sites between

which the level of clockwise transcription increases significantly in a restriction fragment known to bind RNA polymerase) and also the trfB promoter (identified as a region of strong homology to the E. coli concensus promoter sequence lying across the trfB region HincII site which was predicted to lie within the promoter from studies with galK fusions and deletion analysis (27) and confirmed by protection from HincII restriction endonuclease digestion by bound RNA polymerase) (23). These promoter sequences are aligned in Fig. 4 with respect to putative -10 and -35 sequences. A feature of all 3 promoters is the presence of inverted repeat sequences which suggest the presence of protein binding sites. Both the trfA and trfB promoters are identical in this respect and have 2 related but different inverted repeats, one overlapping the -10 sequence, the other upstream from but close to the -35 sequence. The kilB promoter has an identical copy of this latter repeat but shifted to overlap the -10 sequence, Fig. 4. The inverted repeat common to all three promoters we propose as being the site which provides sensitivity to repression by korB while the inverted repeat found only in the trfA and trfB promoters we propose to be associated with trfB/korA/ korD sensitivity. While we do not yet have a good data for regulation of transcription from the trfB promoter, the striking similarities between the trfA and trfB promoters suggest that their regulatory properties are probably similar. So we propose that transcription from the trfB promoter is probably doubly autoregulated by the products of both the trfB/korA/korD locus and the korB locus. These regulatory circuits are summarized in Fig. 5.

Control of RK2 Plasmid Copy Number

Of major interest is the means by which RK2 copy number is regulated. Plasmid copy number is regulated by the frequency of initiation of plasmid replication and, in general, elements that

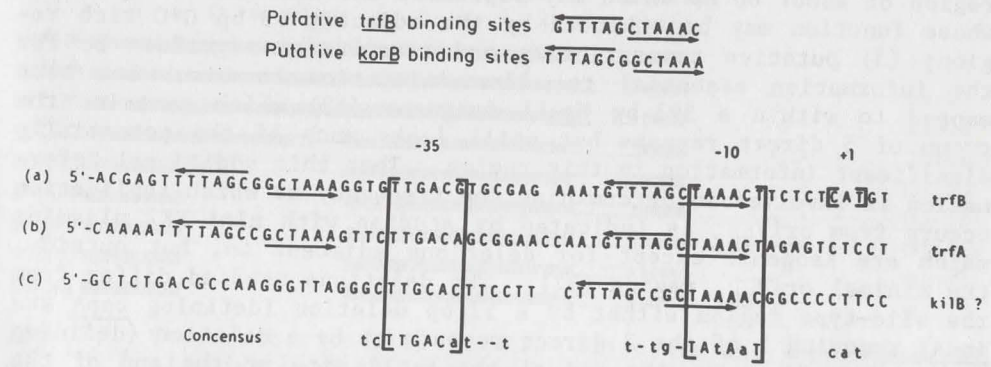


Fig. 4. Sequence of trfA, trfB and kilB promoters showing shared palindromic sequences which may be repressor binding sites.

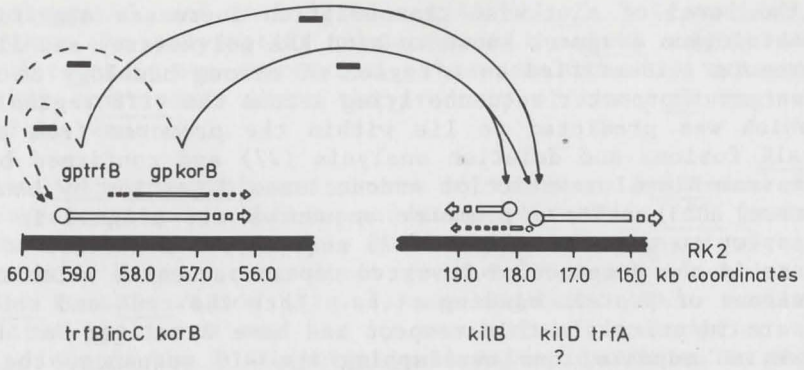


Fig. 5. Summary of established and proposed regulatory circuits in the trfA and trfB regions.

control plasmid copy number have been identified by their ability to interfere with plasmid replication in trans (incompatibility), and by the fact that mutations that alter them in cis may alter plasmid copy number. Since the region of the RK2 origin of vegetative replication contained the first major incompatibility determinant(s) located (30,11) initial efforts to understand copy number control concentrated on this region. The region containing the vegetative replication origin has been sequenced (13,31) and while the elements in and around the replication origin are currently under investigation the significance (and in some cases the reality) of elements inferred from the DNA sequence will become clear only after further work. A summary of this region is shown in Fig. 6. Obvious features in this region are: (1) a series of direct repeats ($TGACA^{CT}_{GA}TGAGGGGCN^{C}_{G}$) of about 17 conserved base pairs which are arranged as a single copy, a group of three and a group of five - in the group of five the repeat unit is 22 or 23 bp; (2) an A+T rich region of about 60 bp which may represent the initiation region and whose function may be enhanced by the adjacent 65 bp G+C rich region; (3) putative transcription and translation signals. So far the information essential to allow initiation to occur has been mapped to within a 393 bp HpaII fragment (12) which contains the group of 5 direct repeats but still lacks much of the potentially significant information in this region. That this additional information is involved in determining the frequency at which replication occurs from $oriV_{RK2}$ is indicated by studies with mini RK2 plasmids which are isogenic except for deletions adjacent to, but outside, the minimal $oriV_{RK2}$ region (32). The deletions studied differ from the wild-type region either by a 57 bp deletion (defining copA and incA) removing 2 of the 3 direct repeats or by a deletion (defining copB and incB) up to the end of the HaeII site at the end of the HaeII $oriV_{RK2}$ fragment close to the BglII fragment and thus removing the single copy of the direct repeat and the beginning of the puta-

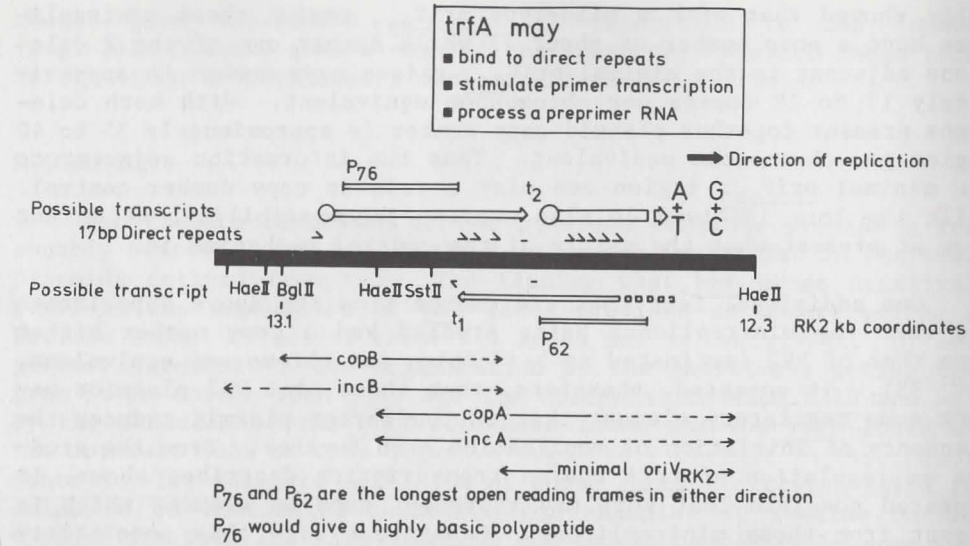


Fig. 6. Organization of $oriV_{RK2}$ region.

tive transcript and open reading frame shown as P_{76}. The minireplicons studied contained the loci trfB, incC, kilD, and trfA, but not kilB or korB. The structure of these plasmids (pCT539, pCT460, pCT543 and pCT461) and their copy numbers, estimated by comparison with pBR322 as an internal control, are shown in Fig. 7. The re-

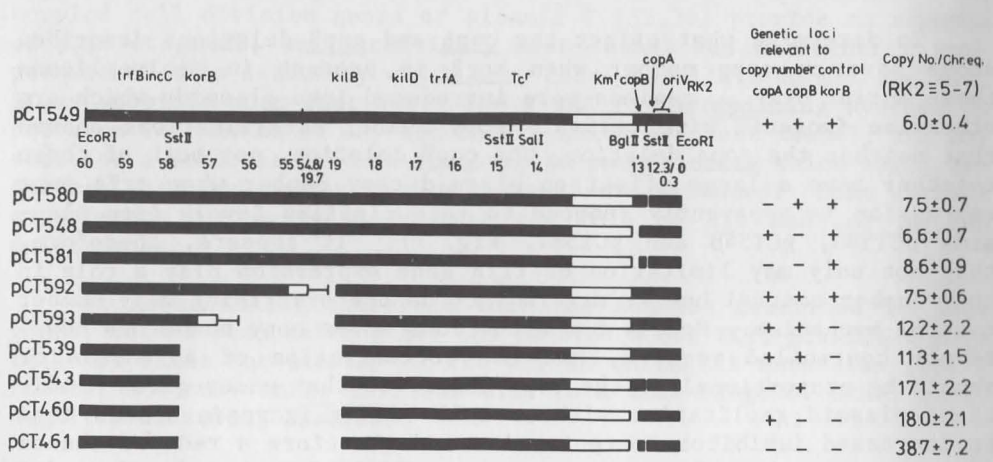


Fig. 7. Copy number control in RK2. Structure and copy number of plasmids used to investigate the importance of copA, copB and korB. RK2 DNA is shown as a thick solid line whose RK2 kb coordinates are shown at the top for pCT549.

sults showed that with a wild-type $oriV_{RK2}$ region these minireplicons have a copy number of about 11 while either one of the 2 deletions adjacent to the minimal $oriV_{RK2}$ raises copy number to approximately 17 to 18 copies per chromosome equivalent. With both deletions present together plasmid copy number is approximately 35 to 40 copies per chromosome equivalent. Thus the information adjacent to the minimal $oriV_{RK2}$ region can play a role in copy number control. While the loci involved do also express incompatibility, we do not know at present what the nature of the control mechanism is.

One additional fact that did emerge from the above experiments was that the minireplicons being studied had a copy number higher than that of RK2 (estimated as 5-7 copies per chromosome equivalent, Ref. 33). It appeared, therefore, that these mini RK2 plasmids may lack some regulatory element that in the parent plasmid reduces the frequency of initiation of replication even further. From the studies on regulation of trfA operon transcription described above, it appeared possible that korB may represent such an element which is absent from these minireplicons. Consistent with this possibility was the fact that korB in trans was found to express incompatibility towards these minireplicons (34). When korB was reintroduced into these minireplicons the copy number was found to drop to a level within or close to the range found for RK2 itself (see pCT549 and pCT592, Fig. 7) (34) while similar manipulations on the plasmid without introducing a functional korB gene (pCT593, Fig. 7) had no significant effect on plasmid copy number. It thus appears that in these minireplicons korB may be further reducing trfA gene expression to a level where trfA gene product concentration has become limiting for replication.

To determine what effect the copA and copB deletions described above have on copy number when korB is present in minireplicons these mutant $oriV_{RK2}$ regions were introduced into plasmids which are otherwise isogenic with pCT549. Copy number determinations showed that neither the copA deletion, the copB deletion, nor both of these together have a large effect on plasmid copy number when trfA gene expression is apparently reduced to rate limiting levels (see plasmids pCT580, pCT548 adn pCT581, Fig. 7). It appears, therefore, that not only may limitation of trfA gene expression play a role in copy number control but it may in fact be the overriding copy number control mechanism. Models for situations where copy number is negatively controlled require that the concentration of an inhibitor should be proportional to the copy number so that under given conditions plasmid replication without cell volume increase should lead to increased inhibitor concentration and therefore a reduced chance of initiation of replication occurring. If korB expression depended solely on the autogenously regulated circuit proposed for transcription from the trfB promoter then its concentration would not be expected to vary in this way. It is worthwhile recalling, therefore, that there seems to be a secondary promoter in the trfB region which should lack the regulatory properties of the trfB promoter and which

should give expression of korB in proportion to copy numbe (Fig. 3). Clearly a knowledge of the exact way in which these regulatory circuits work awaits much more detailed analysis.

DISCUSSION

The results described in the previous sections provide a reasonably comprehensive map of the loci so far identified in the smal plasmids derived from RK2. The finding that kor genes negatively regulate the expression of kil genes provides an understanding o how the lethal effect of these kil genes may be overridden. Of eve greater interest is the organization of the positively acting trf gene in an operon with kilD and its coregulation with kilD and kil by the products of the trfB/korA/korD locus and the korB locus an the apparent role of this negative regulatory circuit in copy numbe control. The detailed mechanism of these control circuits still remains to be elucidated however. For example, it remains uncle whether the trfB and korB products act independently or in some concerted way. In addition we still do not understand the role of inc in these circuits (see Fig. 3 and 4). Recently we have found tha the miniplasmids containing korB are sensitive to incC in trans suggesting that limiting levels of trfA gene expression make replicon sensitive to incC incompatibility. Thus, incC may play an additional role in this replication and maintenance regulatory circuit.

Another intriguing question is what role do the kil genes o RK2 play? Since they are coregulated with trfA it is tempting t suggest that they play a role in the stable maintenance of RK2. Th coupled cell division genes of plasmid F (35,36) provide an exampl of pair of genes, one potentially host lethal and the other a suppressor of this lethality which show close parallels to kil and kor It is possible also that while the F and P1 partitioning systems d not seem to consist of any components which are host lethal, RK2 ma code for a membrane protein involved in partitioning which might interfere with host membrane functions if overproduced. Some curren experiments are designed to test the role of kil and kor in RK maintenance.

It seems likely therefore that the studies described in thi paper, rather than solving all the puzzles about RK2, provide a mor rational basis for studies of the way in which RK2 maintains itsel stably, not only in E. coli but also in a wide range of other gram negative bacterial species.

ACKNOWLEDGMENTS

We thank K. Ellis and P. Barth for communication of result prior to publication; M. Rosenberg for pKO plasmids; R. Schmitt fo Tn1722 derivatives; H. Howell for technical assistance; E. Badge

and B.S. Price for help in preparing the manuscript. V. Shingler was supported by a Medical Research Council (MRC) Studentship for training in research methods. M.A. Cross was supported by a Science and Engineering Research Council (SERC) Quota Award; SERC also provided assistance in his attending this Conference. C.A. Smith was supported by MRC Project Grant No. G80/0374/9CB; M. Pinkney is supported by MRC Project Grant No. G83/0983/8CB; both awarded to C.M. Thomas. The Wellcome Trust and the Royal Society provided assistance for C.M. Thomas to attend this Conference.

REFERENCES

1. Thomas, C.M. (1981) Molecular genetics of broad host range plasmid RK2. Plasmid 5:10-19.
2. Guiney, D.G. (1984) In Plasmids in Bacteria, D.R. Helinski et al., eds. Plenum, New York.
3. Lanka, E., R. Lurz, and J.P. Furste (1983) Molecular cloning and mapping of SphI restriction fragments of RP4. Plasmid 10:303-307.
4. Barth, P.T., and N.J. Grinter (1977) Map of plasmid RP4 derived by insertion of transposon C. J. Mol. Biol. 113:455-474.
5. Barth, P.T., N.J. Grinter, and D.E. Bradley (1978) Conjugal transfer system of plasmid RP4: Analysis by transposon 7 insertion. J. Bact. 133:43-52.
6. Barth, P.T. (1979) PR4 and R300B as wide host-range plasmid cloning vehicles. In Plasmids of Medical, Environmental and Commercial Importance, K.N. Timmis and A. Puhler, eds. Elsevier, North Holland, Amsterdam/New York, pp. 399-410.
7. Lanka, E., and P.T. Barth (1981) Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. J. Bact. 148:769-781.
8. Guiney, D.G., and D.R. Helinski (1979) The DNA-protein relaxation complex of the plasmid RK2: Location of the site specific nick in the region of the proposed origin of transfer. Mol. Gen. Genet. 176:183-189.
9. Guiney, D.G., and E. Yakobsen (1983) Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. Proc. Natl. Acad. Sci., USA 80:3595-3598.
10. Figurski, D., and D.R. Helinski (1979) Replication of an origin-containing derivative of plasmid RK2 dependent upon a plasmid function provided in trans. Proc. Natl. Acad. Sci., USA 76:1648-1652.
11. Thomas, C.M. R. Meyer, and D.R. Helinski (1980) Regions of broad host range plasmid RK2 which are essential for replication and maintenance. J. Bact. 141:213-222.
12. Thomas, C.M., D.M. Stalker, and D.R. Helinski (1981) Replication and incompatibility properties of segments of the origin region of replication of broad host range plasmid RK2. Mol. Gen. Genet. 181:1-7.

13. Stalker, D.M., C.M. Thomas, and D.R. Helinski (1981) Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:8-12.
14. Meyer, R., and D.R. Helinski (1977) Unidirectional replication of the P-group plasmid RK2. Biochim. Biophys. Acta 478:109-113.
15. Thomas, C.M. (1981) Complementation analysis of replication and maintenance functions of broad host range plasmids RK2 and RP1. Plasmid 5:277-291.
16. Schmidhauser, T., M. Filutowicz, and D.R. Helinski (1983) Replication of derivatives of the broad host range plasmid RK2 in two distantly related bacteria. Plasmid 9:325-330.
16a. Schmidhauser, T., and D.R. Helinski (1984) In Plasmids in Bacteria, D.R. Helinski et al., eds. Plenum, New York.
17. Smith, C.A., and C.M. Thomas (1983) Deletion mapping of kil and kor functions in the trfA and trfB regions of broad host range plasmid RK2. Mol. Gen. Genet. 190:245-254.
18. Pohlman, R.F., and D.H. Figurski (1983) Essential genes of plasmid RK2 in Escherichia coli: trfB region controls a kil gene near trfA. J. Bact. 156:584-591.
19. Figurski, D.H., R.F. Pohlman, D.H. Bechhofer, A.S. Prince, and C.A. Kelton (1982) The broad host range plasmid RK2 encodes multiple kil genes potentially lethal to Escherichia coli host cells. Proc. Natl. Acad. Sci., USA 79:1935-1939.
20. Schmitt, R., E. Bernhard, and R. Mattes (1979) Characterization of Tn1721, a new transposon containing tetracycline resistance genes capable of amplification. Mol. Gen. Genet. 172:53-65.
21. Shingler, V., and C.M. Thomas (1984) Analysis of the trfA region of broad host range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. J. Mol. Biol. 175:229-250.
22. McKenney, K., H. Shimatake, D. Court, U. Schmeissher, C. Brady, and M. Rosenberg (1981) A system to study promoter and terminator signals recognized by Escherichia coli RNA polymerase. In Gene Amplification and Analysis. Volume II: Analysis of Nucleic Acids by Enzymatic Methods, J.C. Chirikjian and T.S. Papas, eds. Elsevier/North Holland, Amsterdam, pp. 282-415.
23. Smith, C.A., V. Shingler, and C.M. Thomas (1984) The trfA and trfB promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. Nucl. Acids Res. 12:3619-3630.
24. Smith, C.A., and C.M. Thomas (1984) Nucleotide sequence of the trfA gene of broad host range plasmid RK2. J. Mol. Biol. 175:251-262.
25. Ohlendorf, D.H., W.F. Anderson, and B.W. Matthews (1983) Many gene-regulatory proteins appear to have a similar α-helical fold that binds DNA and evolved from a common precursor. J. Mol. Evol. 19:109-114.
26. Shingler, V., and C.M. Thomas (1984) Transcription in the trfA region of broad host range plasmid RK2 is regulated by trfB and korB. Mol. Gen. Genet. (in press).

26a. Pohlman, R.F., and D.H. Figurski (1983) Conditional lethal mutants of the kilB determinant of broad host range plasmid RK2. Plasmid 10:82-95.
27. Smith, C.A., and C.M. Thomas (1984) Molecular genetic analysis of the trfB and korB region of broad host range plasmid RK2. J. Gen. Microbiol. 130:1651-166.
28. Bechhofer, D.H., and D.H. Figurski (1983) Map location and nucleotide sequence of korA, a key regulatory gene of promiscuous plasmid RK2. Nucl. Acids Res. 11:7452-7469.
29. Meyer, R., and M. Hinds (1982) Multiple mechanisms for expression of incompatibility by broad host range plasmid RK2. J. Bact. 152:1078-1090.
30. Meyer, R. (1979) Expression of incompatibility by derivatives of the broad host range IncP-1 plasmid RK2. Mol. Gen. Genet. 177:155-161.
31. Waters, S.H., P. Rogowsky, J. Grinsted, J. Altenbuchner and R. Schmitt (1983) The tetracycline resistance determinants of RP1 and Tn1721: nucleotide sequence analysis. Nucl. Acids Res. 11:6089-6015.
32. Thomas, C.M., M.A. Cross, A.A.K. Hussain and C.A. Smith (1984) Analysis of copy number control elements in the region of the vegetative replication origin of the broad host range plasmid RK2. EMBO 3:75-63.
33. Figurski, D., R. Meyer and D.R. Helinski (1979) Suppression of ColE1 replication properties by the IncP-1 plasmid RK2 in hybrid plasmids constructed in vitro. J. Mol. Biol. 133:295-318.
34. Thomas, C.M. and A.A.K. Hussain (1984) The korB gene of broad host range plasmid RK2 is a major copy number control element which may act together with trfB by limiting trfA expression. EMBO 3: (in press).
35. Ogura, T., and S. Hiraga (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci., USA 80:4784-4788.
36. Karoui, H., F. Bex, P. Dréze and M. Couturier (1983) Ham22, a mini-F mutation which is lethal to host cell and promotes recA-dependent induction of lambdoid prophage. EMBO 2:1863-1868.

CONTROL OF CHROMOSOME REPLICATION IN BACTERIA

R.H. Pritchard

Department of Genetics
The University of Leicester
Leicester, England

INTRODUCTION

The exciting progress that has recently been made in unravelling the replication control mechanisms that determine the copy numbers of several plasmids has been reviewed and updated by several contributors to this volume. In contrast, the control of chromosome replication in bacteria remains largely a closed book.

As with plasmids, three principal approaches have been used to investigate the control of initiation of chromosome replication in E. coli.

Isolation of Minireplicons

It at one time seemed likely that the isolation of oriC plasmids (1) would open up chromosome initiation control to investigation, but these plasmids have not been as useful as they had promised. One of their more puzzling properties is their failure to express strong incompatibility against the chromosome itself. Thus (2) a stable pBR322 derivative with a copy number of about 30 containing a functional 1.4-kb oriC plasmid as an insert did not significantly alter host-cell size or growth rate. This means that this segment of chromosomal DNA does not code for a trans-acting rate determining effector of chromosome initiation, and makes it unlikely that it contains a target capable of titrating out such an effector coded for by the host.

Interpretation of similar data obtained using larger oriC plasmids (3) is less straightforward because, although the average copy number is 5-10, a significant proportion of the cell population is

plasmid-free. Nevertheless, the survival and growth of the plasmid-containing component of the population surely leads to the same conclusion.

The fact that oriC plasmids do not code for a trans-acting inhibitor (or controlling initiator) of replication is not surprising. There is no a priori reason why the coding sequence should be in close proximity to the initiation site. On the contrary, there are good reasons (4,5) for supposing that such a gene should be located some distance from the initiation site in order to permit synchronous replication of origins in cells with more than one. The apparent absence of a target close to the site of initiation is less easy to understand and suggests at least three possibilities:

(i) The regulatory system which controls the timing of initiation is fundamentally different from corresponding systems controlling plasmid replication. The difficulty is to imagine a control system which does not involve a trans-acting effector and a target site for that effector near the site of initiation, especially since initiation of replication in cells with more than one origin is essentially synchronous.

(ii) A target site for the effector is located on oriC plasmids but the titrating activity of these sites is lethal to the host cell. On this hypothesis oriC plasmids would all be target-site mutants resulting from the demands of the selection technique used to isolate them.

(iii) A third and interesting possibility, which came out of discussions at this conference with G. Churchward, is that after initiation has taken place replication forks stall at a site some distance away and that the primary control of initiation is at this site. OriC plasmids would lack the normal trans-acting initiation control functions and target sites but would, nevertheless, behave as replicons.

Isolation of Initiation-Defective Mutants

So far, none of the genes which mutate to inhibit initiation appear to be the primary control genes which determine the timing of initiation. Thus, in the case of *dnaA*, the most fully studied gene, defective mutants are recessive, indicating a positive function for the gene product, which is consistent with in vitro studies (6). In addition, the concentration of the gene product appears not to be rate-limiting for replication in nonmutant strains (7). These properties are not consistent with the gene product being the primary control gene.

There is also evidence that the primary control functions which determine the timing of initiation are intact in *dnaA* strains.

Thus, at intermediate temperatures, initiation of replication is delayed so that the DNA:mass ratio in the culture is maintained at a lower level than in wild-type strains (8,9). Addition of chloramphenicol to such a culture results in an immediate release from the initiation delay but the origin:mass ratio only rises approximately to the wild-type level. This result suggests that the normal initiation control which sets the DNA:mass ratio is still fully functional in *dnaA* strains.

Incidentally, the release of the *dnaA* block does not occur if protein synthesis is inhibited by amino-acid starvation instead of chloramphenicol, indicating that it is not inhibition of protein synthesis per se which overcomes this block (8,10). However, if amino-acid starvation is performed in a relaxed (*rel*) background the result mimics that obtained with chloramphenicol. This striking result was interpreted in the following way: in *dnaA* mutants there is a failure to synthesize a stable RNA which is necessary for initiation but is not normally rate-limiting. Addition of chloramphenicol, and amino-acid starvation of a *rel* strain, stimulates stable RNA synthesis (11). At intermediate temperatures, this allows a sufficient rate of synthesis of the stable RNA needed for initiation to permit a normal rate of initiation despite the deficiency of *dnaA* gene product.

Isolation of cop Mutants

This has been less rewarding in the case of chromosome replication than it has been in studies on copy-number control in plasmids.

A strain of *E. coli* B/r with a DNA:mass ratio which is half that of typical *E. coli* strains has been found (12). The difference was attributed to a mutation close to, and possibly in the *dnaA* gene.

There have recently been 2 reports of the isolation of several mutants with a 2- to 4-fold increase in DNA:mass ratio (13, 14). All these mutants were found to carry mutations in either *rpoB* or *rpoC* which specify subunits of RNA polymerase. E. Gutzman and A. Jimenez-Sanchez in my laboratory have also isolated a number of thermosensitive *cop* mutants with elevated DNA:mass ratios at high temperatures and these, likewise, appear to carry mutations in *rpoC*.

These are curious findings. Is it conceivable that such a precisely timed discontinuity in the cell cycle as initiation would be controlled by the level of RNA polymerase or a polymerase cofactor? Do these mutations alter RNA polymerase specificity or is the DNA:mass ratio perturbed simply by an alteration in RNA polymerase activity?

To investigate these questions we asked if rifampicin could mimic the effect of these mutations, which have a slightly reduced

RNA polymerase activity. The result is shown in Fig. 1. This shows that sufficient rifampicin to cause a slight reduction in growth rate has an immediate stimulatory effect on DNA synthesis leading to a 2-fold enhancement in the DNA:mass ratio which was maintained over several mass doublings.

We conclude that mutations in RNA polymerase which increase the DNA:mass ratio do so simply as a result of a reduction in the activity of RNA polymerase. Our interpretation of this rather general effect is as follows.

Replication in some plasmids (such as, ColE1, see Ref. 5) is controlled by a negative-feedback loop in which the rate of initiation of each plasmid is inversely proportional to the concentration of an inhibitor, which is a small unstable RNA. Since this is synthesized constitutively there will be a simple proportionality between the concentration of the plasmid and the concentration of the inhibitor and an inverse proportionality between the concentration of the plasmid and its rate of replication.

If chromosome replication were mediated by a similar negative loop, with constitutive synthesis of inhibitor, it would be necessary to assume a much stronger dependence of the probability of initiation on inhibitor concentration than is the case with plasmids like ColE1 in order to account for the very strong dependence of the timing of initiation on cell volume.

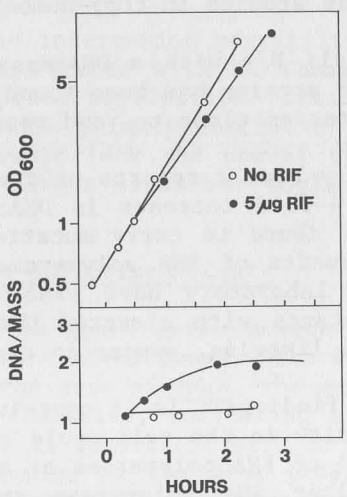


Fig. 1. Mass (absorbance) increase and relative DNA:mass ratio in E. coli LE234 (metB, argH, ilv, rpsl, tna, xyl, mtl, supE) incubated at 42°C in the presence and absence of rifampicin. say 10%.

Consider now the effect of an inhibition of RNA polymerase sufficient to reduce the overall rate of RNA synthesis and growth by say 10%. There would also be a 10% reduction in the rate of inhibitor synthesis. Because of the stronger dependence of initiation on inhibitor concentration this would have a disproportionate effect on the rate of initiation.

If the inhibitor of chromosome replication were a small unstable RNA this might also account for the failure, so far, to isolate thermosensitive copy effect mutants of it.

REFERENCES

1. Yasuda, S., and Y. Hirota (1977) Cloning and mapping of the replication origin of Escherichia coli. Proc. Natl. Acad. Sci., USA 74:5458-5462.
2. Yamaguchi, K., M. Yamaguchi, and J. Tomizawa (1982) Incompatibility of plasmids containing the replication origin of the Escherichia coli chromosome. Proc. Natl. Acad. Sci., USA 79:5347-5351.
3. von Meyenburg, K., F.G. Hansen, E. Riise, H.E.N. Bergmans, M. Meijer, and W. Messer (1978) Origin of replication, oriC, of the Escherichia coli chromosome: Genetic mapping and minichromosome replication. Cold Spring Harbor Symp. Quant. Biol. 43:121-128.
4. Pritchard, R.H. (1978) Control of DNA replication in bacteria. In DNA Synthesis: Present and Future, I. Molineux and M. Kohijama, eds. Plenum, New York.
5. Pritchard, R.H. (1984) Control of DNA replication in bacteria. In The Microbial Cell Cycle, P. Nurse, and E. Streiblova, eds. CRC Press.
6. Fuller, R.S., and A. Kornberg (1983) Purified dnaA protein in initiation of replication at the Escherichia coli chromosomal origin of replication. Proc. Natl. Acad. Sci., USA. 80:5817-5821.
7. Churchward, G., P. Holmans, and H. Bremer (1983) Increased expression of the dnaA gene has no effect on DNA replication in a dnaA+ strain of Escherichia coli. Mol. Gen. Genet. 192:506-508.
8. Orr, E., P.A. Meacock, and R.H. Pritchard (1978) Genetic and physiological properties of an Escherichia coli strain carrying the dnaA gene mutation T46. In DNA Synthesis: Present and Future, I. Molineaux, and M. Kohimaja, eds. Plenum, New York.
9. Hansen, F.G. and K.V. Rasmussen (1977) Regulation of the dnaA product in Escherichia coli. Mol. Gen. Genet. 155:219-225.
10. Lycett, G.W., E. Orr, and R.H. Pritchard (1980) Chloramphenicol releases the block in initiation of chromosome replication in a dnaA strain of Escherichia coli K12. Mil. Gen. Genet. 178:329-336.

11. Shen, V., and H. Bremer (1977) Chloramphenicol induces changes in the synthesis of ribosomal transfer and messenger ribonucleic acids in Escherichia coliB/r. J. Bact. 130:1098-1108.
12. Choung, K.K., E. Estiva, and H. Bremer (1981) Genetic and physiological characterization of a spontaneous mutant of Escherichia coliB/r with aberrant control of deoxyribonucleic acid replication. J. Bact. 145:1239-1246.
13. Rasmussen, K.V., T. Atlung, G. Kersman, G.E. Hansen, and F.G. Hansen (1983) Conditional change of DNA replication control in an RNA polymerase mutant of Escherichia coli. J. Bact. 1564:443-451.
14. Tanaka, M., H. Ohmori, and S. Hiraga (1983) A novel type of E. coli mutants with increased chromosomal copy number. Mol. Gen. Genet. 192:51-60.

STABLE MAINTENANCE OF PLASMID CLO DF13:

STRUCTURAL AND FUNCTIONAL RELATIONSHIPS BETWEEN REPLICATION CONTROL, PARTITIONING, AND INCOMPATIBILITY

H. John J. Nijkamp, Bob van Gemen, Marcel J.J. Hakkaart, Arnold J. van Putten, and Eduard Veltkamp

Department of Genetics, Biological Laboratory
Vrije Universiteit
de Boelelaan 1087
1081 HV Amsterdam, The Netherlands

INTRODUCTION

Nonconjugative plasmids are a very attractive model system for the study of the structural and functional aspects of stable maintenance of replicons in a growing bacterial population. Biochemical and molecular genetic studies have resulted in more insight into the genes and sites involved in the processes responsible for plasmid maintenance such as plasmid replication and its control, accurate partitioning of plasmids over daughter cells, and incompatibility between replicons.

In this chapter, the significance of plasmid-determined processes in the stable maintenance of replicons will be exemplified by the nonconjugative bacteriocinogenic plasmid Clo DF13. We will summarize the structural and functional aspects of Clo DF13 replication control, partitioning, and incompatibility, and we will discuss their inter-relationships.

Plasmid Clo DF13 (5.75 MD) originates from Enterobacter cloacae DF13, but is also stably maintained in Escherichia coli. In the latter host, the plasmid is present to the extent of about 10 copies per cell (1). A genetic and functional map of Clo DF13 (for reviews, see Ref. 2,3) is given in Fig. 1, which includes the relevant genes and sites involved in replication control, partitioning, and incompatibility.

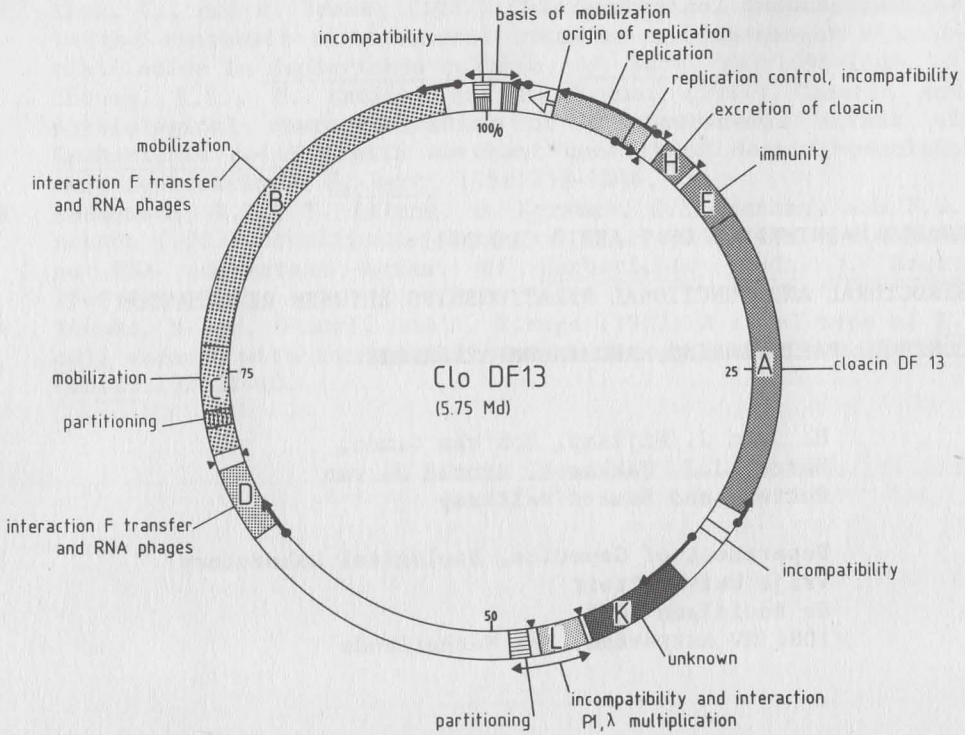


Fig. 1. Genetic and functional map of plasmid Clo DF13. Identified promoters (●) and terminators (▼) as well as the direction of transcription (→) are indicated in the map.

REPLICATION CONTROL

The autonomous replication of nonconjugative plasmids takes place in a controlled manner so that the concentration of a given plasmid is maintained at a constant level in a particular host bacterium grown under defined conditions. Nonconjugative plasmids like Clo DF13 and Col E1 do not code for proteins involved in the replication machinery but they have sequences that meet the requirements for autonomous and controlled replication in dividing cells. They possess determinants that constitute the so-called minimal replicon. The determinants include (i) the origin of replication, (ii) a sequence for the synthesis of RNA involved in priming of DNA synthesis, (iii) overlapping with this latter sequence a gene coding for RNA molecules that control the formation of primer RNA, and (iv) because a minimal replicon is stably maintained, genetic information for accurate partitioning can be expected in that stretch of DNA. In the case of Col E1, the product of the rop gene located outside

the minimal replicon negatively affects the synthesis of primer RNA (5). Both replication control and partitioning are presumed to be the basis of phenomena known as incompatibility (see next sections).

Figure 2 summarizes the relevant features of the Clo DF13 minimal replicon located between 1.8% and 11.5% on the plasmid genome (3). Replication of Clo DF13 starts at a unique sequence, designated oriV and proceeds unidirectionally counter clockwise. Two species of RNA are encoded in the region preceding the replication origin. One species, the preprimer RNA (RNA II), is transcribed from a promoter (P2) about 550 basepairs upstream the origin and terminating beyond it. RNase H processes RNA II at the origin to form a primer upon which DNA polymerase I acts to initiate replication (6). The second RNA species, RNA I, is smaller (105 nucleotides) and is transcribed in the opposite direction to RNA II, starting from promotor P1 and terminating at terminator t1 (7). The coding sequences for RNA I and RNA II overlap and are thus complementary. RNA I negatively controls DNA replication by preventing the processing of RNA II by RNase H (8).

Clo DF13 Copy Control Mutants

A number of plasmid copy control (cop) mutants have been isolated and characterized (9,10,11,12,13). With respect to Clo DF13, 2 different types of replication control mutants can be distinguished. Mutants of the first type were shown to have a nonconditional

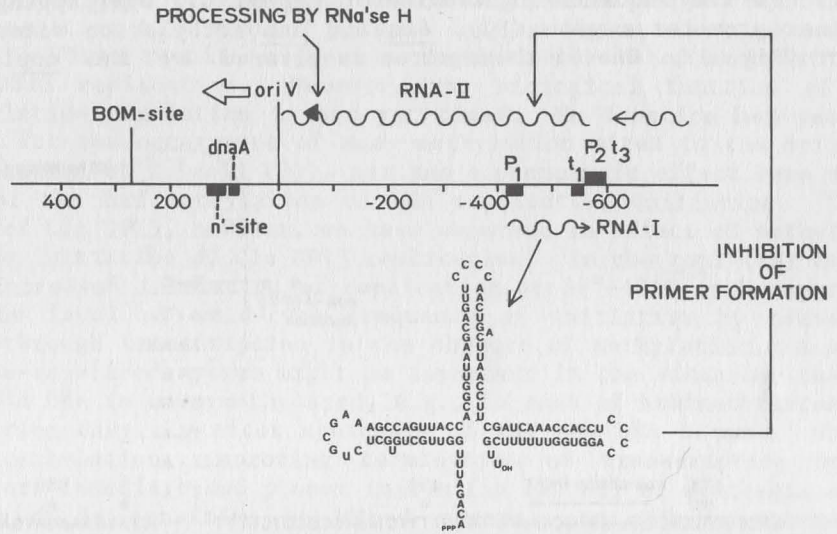


Fig. 2. Structural and functional aspects of the minimal replicon of plasmid Clo DF13.

increase in copy number (9). Sequence analysis revealed that these mutations are located within the region encoding RNA I (11). Based on the location of this type of cop mutations it has been proposed that the target of RNA I is the complementary sequence of RNA II. As shown in Fig. 2, RNA I may adapt a tRNA-like structure with 3 stem-loops. It is assumed that the single-stranded loop structures are involved in the inhibitory activity of RNA I by basepairing with the complementary sequence of RNA II. The second type of replication control mutants is represented by Clo DF13 copl (TS), which is a conditional copy mutant (10). At 30°C the copy number of this mutant is, in comparison with that of the wild-type plasmid, only slightly increased; at 42°C the copy number increases drastically. At this latter temperature the mutant plasmid shows a "runaway" behavior. Here, we report recent data on the nature of this mutation, which is located within a region involved in the initiation of RNA II (P2) and the termination (t3) of the cloacin operon transcript (see Fig. 2). From experiments in which we cloned this region into promoter-study respectively terminator-study systems, we concluded that the Clo DF13 copl (TS) mutation results in a pleiotropic effect, namely, a nonconditional increase in promoter P2 activity and a temperature-dependent decrease in termination efficiency both resulting in an increased synthesis of preprimer RNA. (A. van Putten et al., manuscript in prep.).

Methylation Regulated Clo DF13 Replication

Recently, we obtained evidence that the absence of Dam or Dcm methylation leads to a loss of the runaway behavior of the copl (TS) mutant. In the sequence of terminator region t3, overlapping the preprimer promoter region (P2), dam and dcm methylation sites are present (Fig. 3). One of these sites is altered by the copl (TS)

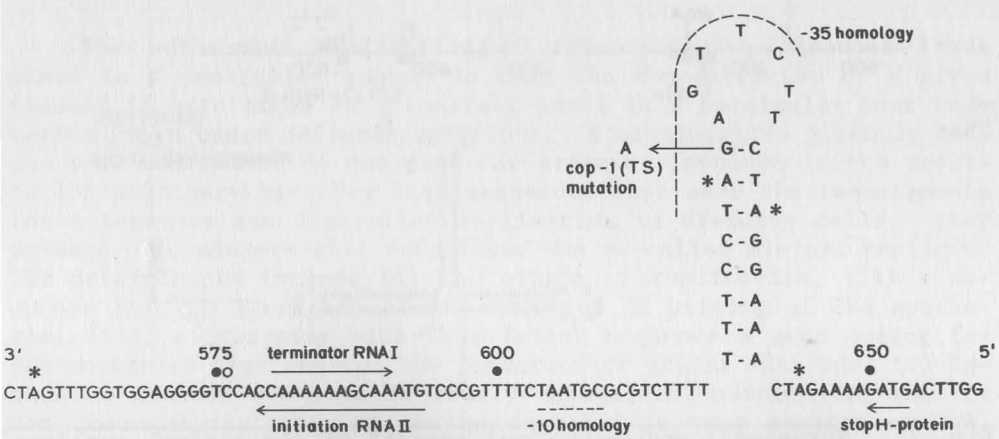


Fig. 3. Nucleotide sequence of the terminator region (t3) of the cloacin operon. Putative dam (*) and dcm (o)methylation sites are indicated.

Tab. 1. Galactokinase (galK) activity of cells harboring the plasmids pKG1800 and pVU402. Cells were grown at 42°C. The galactokinase activity observed in the absence of transcription is approximately 10 U. Pgal: galactose promoter of plasmid pKG1800; Tcopl (TS): fragment containing the mutant cloacin operon terminator (t3).

Plasmid construction		Strain C600 *dam⁺ dcm⁺*	Strain GM48 *dam⁻ dcm⁻*
pKG1800	Pgal galK	613	505
pVU402	Pgal Tcop-1(TS)	196	31

Cells were grown at 42°C. The galactokinase activity observed in the absence of transcription is approximately 10 U.
Pgal: galactose promoter of plasmid pKG1800;
T*cop*1 (TS): fragment containing the mutant cloacin operon terminator (t3).

mutation. In order to study the molecular basis of this phenomenon, we determined the efficiency of termination of the cloacin operon terminator structure in the presence and the absence of methylation. For this purpose, we used plasmid pVU 402 harboring the mutant terminator (t3) in terminator-study plasmid pKG1800. The data obtained are shown in Tab. 1. From these results, we conclude that the absence of Dam or Dcm methylation leads to an increased termination at t3 in the copl (TS) mutant, abolishing read-through transcription and resulting in a reduced plasmid copy number at elevated temperatures. These results suggest a regulatory role of methylation in Clo DF13 replication. However, the biological function of this methylation regulation is not yet clear. No function has yet been found for the occurrence of many methylation sites in the origin of replication of E. coli (31), nor has a phenotypic effect been observed for the Dam methylation on DNA replication initiation. In the case of Clo DF13, however, we have observed an effect of methylation on the initiation of Clo DF13 replication. In the copl (TS) mutant, the increased initiation of replication at 37°-42°C is brought back to the level of wild-type frequency of initiation by preventing read-through transcription in the absence of methylation. A methylation-regulated system might be important in the situation that the plasmid DNA is undermethylated, e.g., in case of hemimethylated DNA, occurring directly after synthesis of the new DNA strand. Whether undermethylation, improving termination of transcription at t3, triggers immediate and proper initiation (at P2) of synthesis of RNA II which is sensitive to RNA I control, or affects interaction of this region with the cell membrane or regulatory proteins, is not clear at the moment. An accurate termination of transcription in the Clo DF13 minimal replicon in case of hemimethylated DNA may be important anyway because the terminator of the RNA I gene, tl, also harbors methylation sites.

PARTITIONING

DNA replication is an absolute requirement for the stable maintenance of a plasmid in a growing cell population. However, plasmid replication alone does not ensure stable inheritance of the plasmid over a great number of cell divisions. To ensure stable inheritance there has to exist, in addition to a proper, controlled replication system, a specific mechanism for accurate partitioning of plasmid molecules at all cell divisions. When there is no specific mechanism for partitioning, and partitioning is an at random process, then it can be demonstrated by mathematical calculations that plasmidless daughter cells rapidly will be generated (14).

It was found for a number of plasmids, including Clo DF13, that certain plasmid DNA regions are involved in the partitioning process. It was shown already in 1978 (15) that certain deletion mutants of Clo DF13 cop plasmids are not stably inherited in the progeny and that the instability of these cop mutants is accompanied by the formation of high amounts of multimeric plasmid DNA molecules. Analysis of these instable Clo DF13 cop mutants revealed that 2 re-

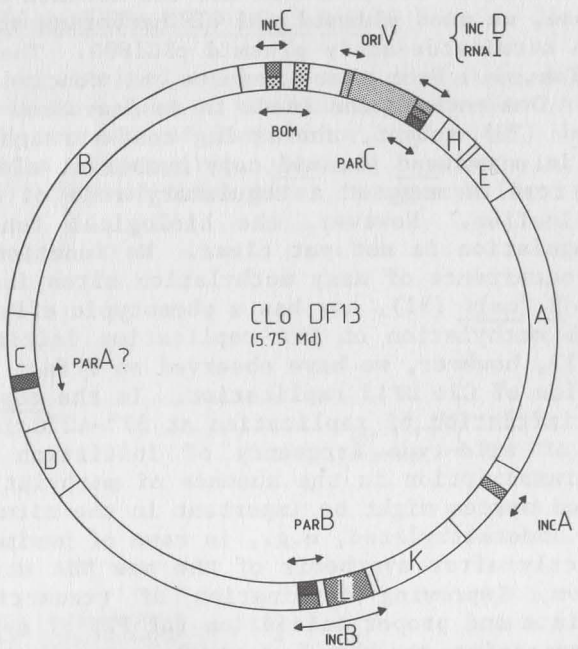


Fig. 4. Genetic information involved in stable maintenance of plasmid Clo DF13: replication control (RNAI), partitioning (par), and incompatibility (inc).

gions on the plasmid genome, designated parA and parB (see Fig. 4) contribute to plasmid maintenance (16). These loci map respectively between 71%-72% and 47%-49% of the Clo DF13 genome. While parA contributes only weakly to plasmid stability, it was found that deletion of the parB region resulted in a strong increase in the generation of multimeric molecules together with an impaired segregation of the plasmids into daughter cells.

Cloning of the site-specific resolution functions of the Tn3-like transposon Tn901 into unstable Clo DF13 derivatives abolished the multimeric forms and reestablished the stable maintenance of these plasmids (16). Apparently, the plasmid par function deleted can be replaced by the resolution functions of transposon TN901. Based on these observations, it was postulated that parB functions in a recombination process that generates the conversion of multimeric DNA, possibly formed during termination of replication, into monomeric DNA (16). Recently, we showed that a recA-independent site-specific resolution process at parB is involved in the maintenance of Clo DF13 cop mutants (17). We found that site-specific resolution occurs if a Clo DF13 plasmid contains 2 parB loci in an intramolecular direct orientation. A specific Clo DF13 sequence within the parB region acts as a site for a site-specific recombination process involved in the resolution of multimeric molecules. This sequence is capable of forming an intra-strand secondary structure (see Fig. 5) because of extensive internal symmetry. A similar system has been described for the stringently replicating phage P1 (18). For a comparison of the site-specific recombination functions of Clo DF13 (parB), P1 (loxP), λ (att site), and of tn3/γδ (res site) see Hakkaart et al. (17).

Clo DF13 gene L, located in the direct vicinity of parB (19) is not essential for parB functioning. However, our genetic data indicate that the transcription from the gene L containing operon into parB is required (17). We have suggested that termination of transcription in the parB site at the stem-loop structure might be a crucial step (20).

The parB resolution system has a number of surprising features: (i) the parB resolution site is located outside the minimal replicon of Clo DF13, (ii) the parB resolution system is required for stable maintenance of Clo DF13 cop mutants, but not for Clo DF13 plasmids with a wild-type number of plasmid copies, and (iii) there is no evidence at all for a crucial and exclusive role of a plasmid-specified gene product required for site-specific recombination at the parB site.

A Clo DF13 cop^+ minimal replicon is stably maintained for at least 100 cell generations, indicating that an active partitioning mechanism is involved. Thus far, a partitioning system exclusively for the Clo DF13 minimal replicon has not been identified.

The difference between wild-type plasmids and cop mutants, with respect to their dependence on parB for stable maintenance, can be explained by assuming that dissociation of multmeric DNA can be performed by 2 systems, a parB-dependent and a parB-independent system. The parB-independent system, likely located in the minimal replicon, should be either a recA-independent, intramolecular recombination process or a site-specific recombination process occurring at an as yet unknown resolution site(s). The parB-independent system might only be capable of resolving a limited number of multimeric molecules during cell growth. Due to this limited capacity, an increase in the frequency of initiation of replication due to a cop mutation will result in the accumulation of multimeric DNA unless the parB resolution function is present on the plasmid. From all our studies we could not obtain any evidence for the crucial and exclusive role of a plasmid-specified gene product, involved in the resolution process. Therefore, we postulate that recombination enzymes encoded by the host are involved in this process. In this respect it should be mentioned that in a recE activated host, both $parB^+$ and $parB^-$ plasmids are stably maintained (16). It is known that in recE-activated cells the interconversion between multimeric and monomeric plasmid molecules is drastically stimulated due to the action of exonuclease VIII (22). In addition, all Clo DF13 $cop^- par^+$ plasmids regenerate multimeric plasmid DNA molecules and are, therefore, not stably maintained in the E. coli host mutant, W5445 ZH37, isolated by P. Derbyshire.

Further observations (21) that may be relevant to Clo DF13 stability are the following: (i) 2 Clo DF13 replicons of exactly the same size but different in their antibiotic-resistance markers have an equal chance to colonize the daughter cells upon cell division, resulting in an equal distribution of the 2 plasmids in the cell population, (ii) in case of cells harboring Clo DF13 replicons that differ in size, the smaller plasmid is gradually lost from the cell population, (iii) in the case of cells harboring Clo DF13 replicons that differ in copy number, the low copy number plasmid is also lost from the cell population, and (iv) the effect of plasmid copy number is dominant over the effect of plasmid size.

INCOMPATIBILITY

Plasmids that are unable to coexist in the same bacterial cell are designated incompatible plasmids. Naturally occurring incompatible plasmids invariably have some sequence homology. This sequence homology is structurally and functionally related at least to essential features of plasmid maintenance: replication control and partitioning (see next section).

For several plasmids it has been possible to map loci for incompatibility. We have examined the incompatibility properties of

plasmid Clo DF13 and we were able to identify and localize 4 different incompatibility (inc) regions on the Clo DF13 genome (23). The map positions of these regions designated incA, incB, incC, and incD are shown in Fig. 4. We have studied the contribution of each of the 4 inc regions, separately as well as in combination with each other, to the incompatibility of 2 plasmid replicons. We found that: (i) plasmid incompatibility can be evoked by the incD region independently of the other inc regions; (ii) the incA, incB, and incC regions do not function on their own; (iii) the function of both incA and incC in plasmid incompatibility is dependent on the presence of the incB region (gene L) (base sequence analysis indicates that incA as well as incC is located in regions not coding for a gene product); and (iv) the incC region only appears to function when the incD region is present in cis.

Based on these observations we propose a distinction between the incompatibility evoked by the incD region (arbitrarily designated type I incompatibility) and the incompatibility evoked by the other Clo DF13 inc regions, incA, incB, and incC (designated type II incompatibility) (23). We will discuss the 2 incompatibility systems in the context of replication control and partitioning in the next section.

RELATIONSHIPS BETWEEN REPLICATION CONTROL, PARTITIONING, AND INCOMPATIBILITY

Incompatibility is a description of a phenomenon: the noncoexistence of 2 replicons in the same cell. This noncoexistence can be regarded as the consequence of fundamental mechanisms for the stable maintenance of plasmids in dividing cells, e.g., replication control and partitioning. We will discuss the structural and functional relationships between the 2 Clo DF13 incompatibility systems, Clo DF13 replication control, and partitioning.

Type I Incompatibility (incD)

The incD region overlaps the Clo DF13 minimal replicon, including the copy control region. The incD exerts the so-called type I incompatibility (23). The favored model for this type of incompatibility is that of Pritchard (24), further developed by others (14, 25), which states that the initiation of replication is controlled by a cytoplasmic repressor substance, the specific activity of which is linked to cell growth (24). Two incompatible plasmids have identical repressors and repressor target sites. Nonconjugative plasmids produce RNA I as replication repressor and their target sites are likely to be the complementary RNA II molecules (8). Copy control mutants that have been located in the RNA I gene (9,10,12, 13) alter repressor and target site in a complementary manner and both have properties different from the wild-type repressor and

target. This might explain why these types of copy control mutants have altered incompatibility properties (8,13,23). These findings fit the model in which the incD region evokes incompatibility at the level of replication control by the action of the trans-acting RNA I repressor, as predicted by the repressor dilution model of Pritchard (24).

A Clo DF13 minimal replicon is stably maintained in dividing cells and therefore this region may also contain information for accurate partitioning. No partitioning system has been identified yet for the Clo DF13 minimal replicon, but we observed that part of the RNA I gene (tentatively designated parC) has a significant nucleotide sequence homology with the parA locus, which is located in one of the mobilization genes, gene C (21). The extent of homology is illustrated in Fig. 5. In contrast to parB, parA contributes only weakly to the stable maintenance of Clo DF13. Therefore, the question is justified whether or not parA is a real partitioning site. It might be a secondary site just because of some sequence homology with a primary par site. If it turned out that parC within the RNA I gene were a partitioning site then incD incompatibility could be the result of 2 mechanisms: replication control and partitioning, 2 mechanisms that may overlap or interact not only structurally but also functionally. The minimal replicon possibly contains also a membrane binding site for replication. This site and a partitioning site are not necessarily the same.

Type II Incompatibility (incA, incB, and incC)

The functioning of incA as well as incC depends on the presence of the incB region (23). From previous studies (27,30) it is known that the gene coding for protein L is located within the 45%-50% region of Clo DF13. Protein L is an inner membrane protein (28) and has a MW of 9.9 Kd (20). Sequence analysis of the incA (29) and the incC (20) region as well as analysis of the proteins produced by Clo DF13 (27) suggests that these inc loci are located within a DNA region not coding for any protein. Taking these observations in account, we have proposed that the incA, incB, and incC form an additional incompatibility system (designated type II incompatibility) that can be explained by a model which is similar to the membrane attachment site model of Jacob et al. (26). We postulated (23) that protein L, product of the gene L in the incB region, is required for the attachment of the plasmid to the cell membrane. This attachment protein facilitates the binding between specific sites, as incA and incC, and the appropriate membrane sites. The inhibition of the multiplication of double-stranded DNA phages by the gene L protein (27) can be the result of the same type II incompatibility mechanism.

The underlying mechanism for the type II incompatibility is likely, at least in part, the partitioning mechanism. The parB site is part of the incB locus. A nucleotide sequence in parB (Fig. 6)

located next to gene L (see Fig. 4) is involved in the site-specific recombination involved in the resolution of multimeric plasmid molecules. One of the possible explanations for the generation of plasmidless cells in case of parB⁻ Clo DF13 cop mutants could be that the proper partitioning of plasmids at cell division is disturbed in case of multimeric molecules; in other words, the multimeric molecules "clog up" the segregation system. At the moment we don't know whether the plasmid instability results from an impaired active partitioning process or from a strongly reduced copy number in an ad random partitioning system. Protein L is not required for multimer resolution. However, transcription from the KL operon into the parB site is necessary. It is not fully excluded that the L protein plays a role in resolution. In Clo DF13 L⁻ mutants the function of the L protein could be replaced by a host protein with a similar function.

The incA locus is located at about 32% of the Clo DF13 genome. This region is strongly involved in gene expression. Both the promoter for the cloacine operon and the promoter for the KL operon are located in that area (20,30). binding sites for the cAMP-CRP complex and lexA protein can be found there as well (30). A nucleotide sequence in the incA region permits the formation of a stem-loop structure as is illustrated in Fig. 6.

In our incompatibility type II model, incA is attached to the membrane with the help of protein L. The natural function of this membrane attachment is unknown. It may play a role in the control of the expression of the KL region, or it may play a more direct role in the site specific recombination. Recently, we have obtained evidence that other sites than parB are involved in recA-independent site-specific recombination (A. van Putten, unpub. results). The nature of this recA independent recombination process is subject to present investigation.

The incC locus, located at 97% of the Clo DF13 genome, overlaps a part of the cis-acting bom site (basis of mobilization). Inactivation of incC by transposon insertion prevents mobilization of Clo DF13 (23). The incC locus does not code for any obvious polypeptides (29) but it contains a nucleotide sequence that permits the formation of a stem-loop structure, very similar to that of the incA locus (see Fig. 6).

About the natural function of incC, one can only speculate at the moment. The incC site may have, after attachment to the membrane by the L protein, either a regulatory function in the initiation or termination of transfer replication or a partitioning function. As partitioning site it may be involved in partitioning of plasmid molecules over daughter-cells or may be involved in partitioning of single-stranded DNA molecules over the conjugating acceptor and donor cell during the initial phase of transfer replication. Under nonconjugative conditions the incC site will not be

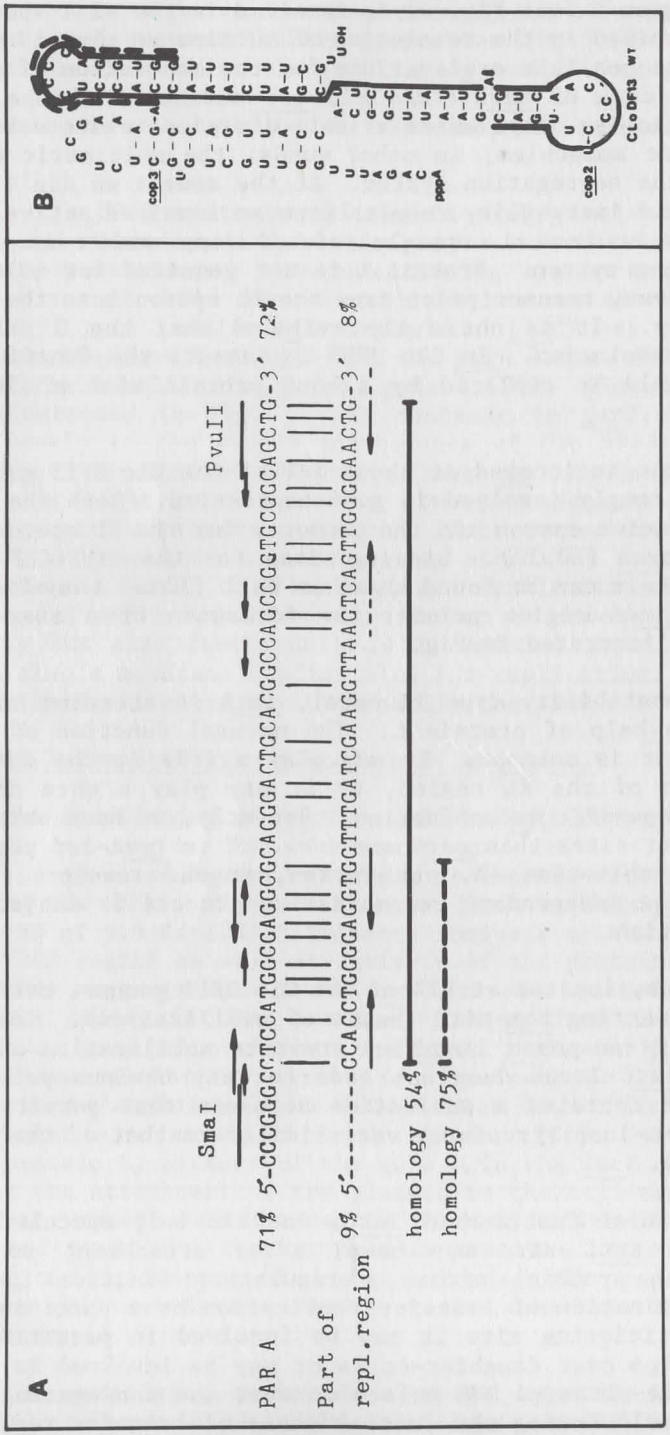


Fig. 5. Nucleotide sequence homology study. A. Nucleotide sequence (5'-3') of part of the parA region of Clo DF13 as determined, and part of the minimal Clo DF13 replicon (11). Marked are: SmaI and PVUII restriction sites. Arrows indicate regions of internal symmetry. Indicated is the region with 54% (-) and 75% (---) sequence homology. B. Nucleotide sequence of the RNA I repressor molecule (11) of Clo DF13. The regions of homology with parA are indicated. (see Fig. 5A).

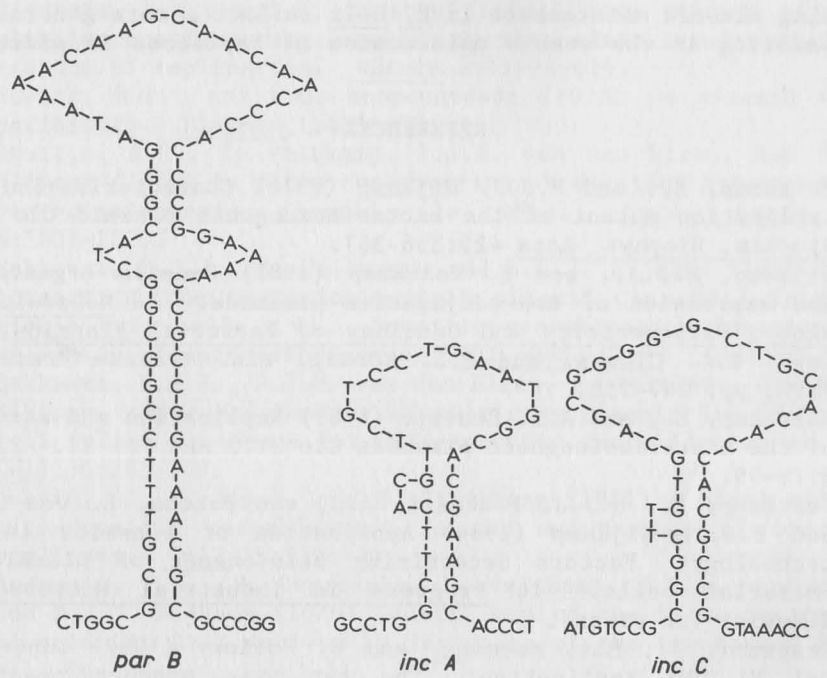


Fig. 6. Possible stem-loop structures within respectively the parB, incA, and incC region of plasmid Clo DF13.

functional in partitioning of single-stranded molecules because of the absence of signals for the initiation of transfer replication, but may be still susceptible for certain partitioning proteins.

A requirement for the incC functioning in incompatibility is the presence in cis of the minimal replicon. Likely, transcription (or replication) from this region into the incC locus is necessary. The stem-loop structure in incC, together with transcription, might be necessary for incC functioning.

In this chapter we have summarized the present knowledge of factors determining the stable maintenance of plasmid Clo DF13 in a growing bacterial cell population. We have discussed the structural and functional aspects of Clo DF13 replication control, partitioning, and incompatibility. In addition, attention is given to the structural and functional relationships between replication control, partitioning, and incompatibility.

Further studies are necessary to unravel the molecular basis of stable inheritance of plasmid DNA not only in E. coli but also in other microorganisms. It might be, however, that the elements de-

ermining plasmid maintenance in E. coli reflect a more general system operating in the stable maintenance of replicons in microorganisms.

REFERENCES

1. Veltkamp, E., and H.J.J. Nijkamp (1976) Characterization of a replication mutant of the bacteriocinogenic plasmid Clo DF13. Biochim. Biophys. Acta 425:356-367.
2. Nijkamp, H.J.J., and E. Veltkamp (1981) Genetic organization and expression of non-conjugative plasmids. In Molecular Biology, Pathogenicity and Oecology of Bacterial Plasmids, S.B. Levy, R.C. Clowes, and E.L. Koenig, eds. Plenum Press, New York, pp. 247-258.
3. Veltkamp, E., and A.R. Stuitje (1981) Replication and structure of the bacteriocinogenic plasmids Clo DF13 and Col El. Plasmid 5:76-99.
4. Veltkamp, E., M.J.J. Hakkaart, A.J. van Putten, B. van Gemen, and H.J.J. Nijkamp (1984) Application of plasmids in gene technology: Factors determining maintenance of plasmids in bacterial cells. In Progress in Industrial Microbiology, Elsevier (in press).
5. Cesareni, G., M.A. Muesing, and B. Polisky (1982) Control of Col El DNA replication: The cop gene product negatively affects transcription from the replication primer promoter. Proc. Natl. Acad. Sci., USA 79:6313-6317.
6. Itoh, T., and J. Tomizawa (1980) Formation of an RNA primer for initiation of replication of Col El DNA by ribonuclease H. Proc. Natl. Acad. Sci., USA 77:2450-2454.
7. Stuitje, A.R., E. Veltkamp, J. Maat, and H.L. Heyneker (1980) The nucleotide sequence surrounding the replication origin of the cop3 mutant of the bacteriocinogenic plasmid Clo DF13. Nucl. Acids Res. 8:1459-1474.
8. Tomizawa, J., T. Itoh, G. Selzer, and T. Som (1981) Inhibition of Col El RN primer formation by a plasmid-specified small RNA. Proc. Natl. Acad. Sci., USA 78:1421-1425.
9. Kool, A.J., and H.J.J. Nijkamp (1974) Isolation and characterization of a copy mutant of the bacteriocinogeic plasmid Clo DF13. J. Bact. 120:569-578.
0. Andreoli, P.M., R.A. Brandsma, E. Veltkamp, and H.J.J. Nijkamp (1978) Isolation and characterization of a Clo DF13::Tn901 plasmid mutant with a thermosensitive control of DNA replication. J. Bact. 135:612-621.
1. Stuitje, A.R., C.E. Spelt, E. Veltkamp, and H.J.J. Nijkamp (1981) Identification of mutations affecting replication control of plasmid Clo DF13. Nature 290:264-267.
2. Muesing M., J. Tamm, H.M. Shepard, and B. Polisky (1981) A single base-pair alternation is responsible for the DNA overproduction phenotype of a plasmid copy-number mutant. Cell 24:235-242.

13. Lacatena, R.M., and G. Cesareni (1981) Base pairing of RNA I with its complementary sequence in the primer transcript inhibits Col El replication. Nature 294:623-626.
14. Novick, R.P., and F.C. Hoppensteadt (1978) On plasmid incompatibility. Plasmid 1:421-434.
15. Stuitje, A.R., E. Veltkamp, P.J.M. van den Elzen, and H.J.J. Nijkamp (1978) In vitro construction of deletion mutants of the bacteriocinogenic plasmid Clo DF13. Nucl. Acids Res. 5:1801-1820.
16. Hakkaart, M.J.J., E. Veltkamp, and H.J.J. Nijkamp (1982) Maintenance of the bacteriocinogenic plasmid Clo DF13 in Escherichia coli cells. II. Specific recombination functions involved in plasmid maintenance. Mol. Gen. Genet. 188:338-344.
17. Hakkaart, M.J.J., P.J.M. van den Elzen, E. Veltkamp, and H.J.J. Nikjamp (1984) Maintenance of multicopy plasmid Clo DF13 in E. coli cells: Evidence for site-specific recombination at parB. Cell 36:203-209.
18. Austin, S., M. Ziese, and N. Steinberg (1981) A novel role for site-specific recombination in the maintenance of bacterial replicons. Cell 25:729-736.
19. Andreoli, P.M., N. Overbeeke, E. Veltkamp, J.D.A. van Embden, and H.J.J. Nijkamp (1978) Genetic map of the bacteriocinogenic plasmid Clo DF13 derived by insertion of the transposon Tn901. Mol. Gen. Genet. 160:1-11.
20. Van den Elzen, P.J.M., M.J.J. Hakkaart, A.J. van Putten, H.H.B. Walters, E. Veltkamp, and H.J.J. Nijkamp (1983) Structure and regulation of gene expression of a Clo DF13 plasmid DNA region involved in plasmid segregation and incompatibility. Nucl. Acids Res. 11:8791-8808.
21. Hakkaart, M.J.J., B. van Gemen, E. Veltkamp, and H.J.J. Nijkamp (1984) Maintenance of multicopy plasmid Clo DF13: III. Role of plasmid size and copy number in partitioning. Mol. Gen. Genet. (in press).
22. Fishel, R.A., A.A. James, and R. Kolodner (1981) RecA-independent general genetic recombination of plasmids. Nature 294:184-186.
23. Hakkaart, M.J.J., J.G. Wesseling, E. Veltkamp, and H.J.J. Nijkamp (1982) Maintenance of the bacteriocinogenic plasmid Clo DF13 in Escherichia coli cells: I. Localization and mutual interactions of four Clo DF13 incompatibility regions. Mol. Gen. Genet. 186:531-539.
24. Pritchard, R.H., P.T. Barth, and J. Collins (1969) Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19:263-297.
25. Hashimoto-Gotoh, T., and K.N. Timmis (1981) Incompatibility properties of Col El and pMB1 derivative plasmids: Random replication of multicopy replicons. Cell 23:299-238.
26. Jacob, F., S. Brenner, and F. Cuzin (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329-348.

27. Van de Pol, H., E. Veltkamp, and H.J.J. Nijkamp (1980) Genetic information of the bacteriocinogenic plasmid Clo DF13 involved in the inhibition of the multiplication of double stranded DNA phages. Mol. Gen. Genet. 178:535-540.
28. Van de Pol, H. (1980) The bacteriocinogenic plasmid Clo DF13: Mobilization and phage interaction. Thesis, Vrije Universiteit, Amsterdam.
29. Snijders, A., A.J. van Putten, E. Veltkamp, and H.J.J. Nijkamp (1983) Localization and nucleotide sequence of the bom region of Clo DF13. Mol. Gen. Genet. 192:444-451.
30. Van den Elzen, P.J.M., J. Maat, H.H.B. Walters, E. Veltkamp, and H.J.J. Nijkamp (1982) The nucleotide sequence of the bacteriocin promoters of plasmid Clo DF13 and Col E1: Role of lexA repressor and cAMP in the regulation of promotor activity. Nucl. Acids Res. 10:1913-1927.
31. Sugimoto, K., A. Oka, H. Sugisaki, M. Takanami, A. Nishimura, Y. Yasuda, and Y. Hirota (1979) Nucleotide sequence of E. coli K12 replication origin. Proc. Natl. Acad. Sci., USA 76:575-579.

REPLICATION CONTROL FOR PT181, AN INDIRECTLY REGULATED PLASMID

Richard P. Novick, Steven J. Projan, C. Chandra Kumar, Stephen Carleton, Alexandra Gruss, Sarah K. Highlander, and John Kornblum

Public Health Research Institute
of the City of New York, Inc.
New York, New York 10016

SUMMARY

PT181 is a fully sequenced Staphylococcus aureus plasmid whose size is 4,437 bp. It specifies tetracycline resistance and has a copy number of about 22 per cell in exponentially growing cultures. The functional organization of the pT181 replicon is centered around the coding sequence for a 35-kd protein, RepC, that is absolutely required for replication of the plasmid. The replication origin is contained within the repC coding sequence and the region immediately 5' to the RepC start is involved in control of the plasmid replication rate. PT181 replication is controlled at the level of RepC synthesis by a negative regulatory system that is functionally similar to that of the ColE1 and IncFII plasmids of Escherichia coli. The pT181 control circuit involves 2 short transcripts, RNA I and RNA II, that are transcribed from the region specifying the 5' end of the untranslated repC mRNA leader and in the opposite direction. These are referred to as countertranscripts. The countertranscripts regulate RepC synthesis by a mechanism that probably involves interaction with the repC mRNA leader in a manner that interferes with translation. Both of the countertranscripts seem to be necessary for normal replication control; their separate roles remain unclear.

Unlike plasmids of the ColE1 and IncFII groups, plasmids such as ColE1 are considered to have direct regulation of replication because the inhibitory element of the copy control circuit directly inhibits the initiation of replication. Plasmids such as pT181 are considered to have indirect regulation of replication because the product of the regulated step, RepC, is trans-active. Plasmids of the IncFII type are considered to have direct regulation of

eplication because the product of the regulated step, RepA is cis-ctive. The analysis of pT181 replication physiology has illustrat-d 2 important differences between directly and indirectly regulated lasmids: a) for directly regulated plasmids, copy mutants specify-ng a normal inhibitor substance but an inactive target site exclude he wild-type or recessive mutants by directly interfering with heir replication. Analogous mutants of indirectly regulated plas-ids coexist readily with the wild-type and all mutants (although hey do manifest segregational incompatibility) because the Rep pro-ein is always shared by all plasmids in the cell, regardless of its ource. b) Mutations of directly regulated plasmids in the region here target transcript and countertranscript overlap may give rise o totally new incompatibility groups because they engender indepen-lently self-correcting copy pools. Such mutants for indirectly egulated plasmids never give rise to new incompatibility groups, even if they have totally different inhibitor-target recognition pecificities because they do not engender independently self-orrecting copy pools due to the mutual availability of the Rep rotein.

These considerations lead to a general view of the basis of lasmid incompatibility that is somewhat broader than can be gained from a consideration of directly regulated plasmids alone.

INTRODUCTION

The details of replication control systems are quite variable from plasmid to plasmid and their comparative analysis has revealed certain basis principles. One of these principles is that the characteristics of the control system are dependent in an important way on the point in the initiation pathway at which the controls act. If the inhibitor acts directly at the initiation step, the regulation is referred to as direct; if the inhibition is of the synthesis of a trans-acting factor required for replication, the regulation is referred to as indirect. Of the 3 groups of plasmids whose replication control mechanisms are best understood, namely the ColE1 and IncFII groups of Escherichia coli and the Inc^{3} group of Staphylococcus aereus, the first 2 have direct regulation, the third, indirect. All 3 groups utilize regulatory circuits of the inhibitor-target type in which the inhibitor is an RNA molecule referred to as a countertranscript. Other well-studied plasmids such as F, R6K, P1, and RK2 utilize a regulatory mechanism that involves binding of a trans-acting replication factor (the initiator) to sets of directly repeated nucleotide sequences (iterons) at or near the replication origin. These initiator-binding mechanisms are probably of the direct variety; however, from the available data, one cannot yet be certain. In this report, we describe the characteristics of the indirect type of regulation, as exemplified by pT181, and contrast these to the direct type as exemplified by the ColE1 family and by R1/NR1/R6-5.

THE pT181 REGULATORY CIRCUIT

pT181 is the prototype of a large and widely dispersed family of small, multicopy staphylococcal plasmids. It specifies inducible tetracycline resistance, has a molecular length of 4,437 bp, and is maintained at a copy number of about 22 in exponential cells. It is inherited stably in S. aureus but unstably in Bacillus subtilis, although it is maintained at approximately the same copy number in both species. The entire nucleotide sequence of the plasmid has been determined (2) and the origin of the numbering sequence is the unique PvuI site (see Fig. 1). The essential replication functions are located in a region of approximately 1.5 kb, between positions 3500 and 500. A region that is required for stability has recently been identified between positions 500 and 700 (A. Gruss and R. Novick, unpub. data). PT181 specifies a trans-active protein, RepC, that is absolutely required for the replication of the plasmid and is specific for plasmids of the Inc^{3} group (3). The determinant for this protein, repC, has been shown by genetic means to correspond to a 939-bp open reading frame extending from bases 3646 to 151 and reading counterclockwise as the map is conventionally drawn (3). We

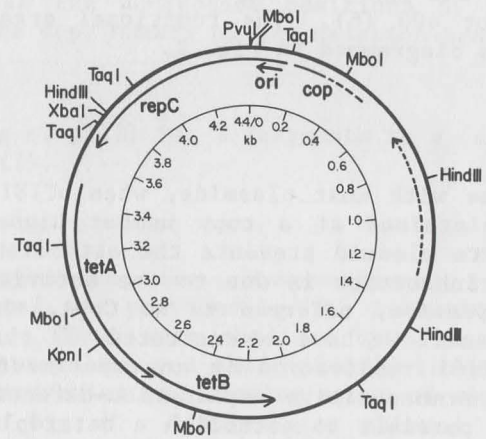


Fig. 1. pT181 map. Kilobase coordinates originate at the Pvu I site at 12:00 and are read in the clockwise direction. Nucleotide positions are based upon the known sequence as determined by Khan and Novick (2). Solid arrows labeled repC, tetA, and tetB indicate open reading frames based on the sequence and known to encode functional proteins. The dashed arrow indicates an open reading frame that is not known to encode any polypeptide. "Ori" indicates the approximate outer limits of the replication origin and the direction of replication (5). "Cop" indicates the region in which the known elements of the replication control system are located (8).

ave shown previously that pT181 is capable of semiconservative replication in cell-free extracts of S. aureus strains containing the plasmid but not in extracts of strains containing no plasmid or any other plasmid (4). The activity of pT181-containing extracts was shown to be due to a plasmid-encoded protein which is probably RepC.

PT181 replicates unidirectionally from a single origin located between nucleotides 15 and 157 (5). Replication is in the counterclockwise direction (see Fig. 1); additionally, all of the known and potential polypeptide coding sequences are also transcribed in the counterclockwise direction. Thus, there is a strong counterclockwise directionality to the general flow of information around the plasmid, which may reflect some degree of coupling between replication and transcription.

Elements of the replication control system for pT181 are located in the region immediately 5' to the RepC coding sequence. As currently understood, this region occupies at least 300 bp and contains the promoters for 4 transcripts: 2 left tandem starts at positions 339 and 410 are used for the repC mRNA and the 2 transcripts are referred to as RNA III and RNA IV, respectively. One right start at position 246 is used for 2 countertranscripts referred to as RNA I and RNA II. The former terminates at about 340, the latter at about 400 (6). The functional organization of the essential region is diagrammed in Fig. 2.

The CopA function

As is the case with most plasmids, when pT181 is cloned to a vector that is maintained at a copy number higher than that of pT181, the composite plasmid prevents the establishment of autonomous pT181. This inhibition is due to the activity of a negative regulator of replication, referred to as CopA, whose activity is gene-dosage dependent. We have demonstrated (7) that CopA activity directly blocks pT181 replication in an experiment in which pT181 was cloned to a thermosensitive replication-defective (Tsr) mutant of pE194. It was possible to establish a heteroplasmid containing pT181 and this latter cointegrate by performing the cross at an elevated temperature. As soon as the temperature was lowered, however, the Tsr replicon recovered its normal copy number and replication of the pT181 replicon was blocked. This experiment is shown in Fig. 3. Using the pT181::pE194 cointegrate, we mapped the position of the inhibitory activity by generating deletions by exonuclease III digestion of Pvu I-linearized DNA in vitro. These results were clear: all of the deletion-bearing plasmids were either as effective as wild-type in excluding pT181 or totally ineffective. The map positions of the deletions are shown in Fig. 4 and indicate that CopA is encoded in the region 5' to the repC start. The inhibitor gene was further localized to the region between positions 158 and

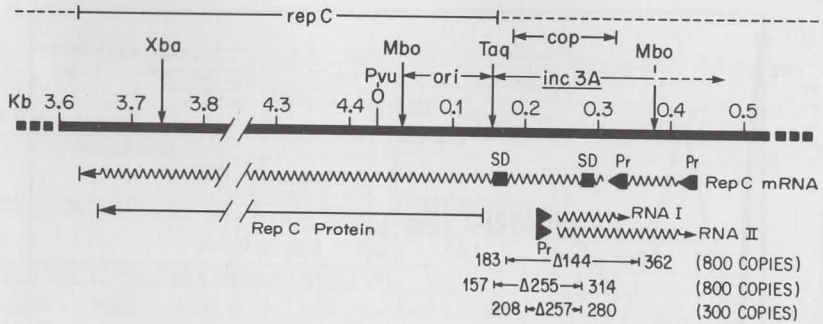


Fig. 2. pT181 regulatory region. Figures are in kilobases, referring to the coordinate system indicated in Fig. 1. Wavy lines indicate known transcripts. For all 4 transcripts shown, the start sites are known and the termination sites are approximated (4). "repC" indicates the extent of the repC gene based on its coding sequence. "Inc3A" refers to the map location of the negative regulatory element of the copy control system, copA, that is responsible for interference in trans with replication of sensitive plasmids. SD = Shine-Dalgarno sequence; Pr = promoter. Below the map are shown the extents of 3 spontaneous deletions, given as the nucleotide positions of their ends, along with the copy number of the deletion-bearing plasmids.

570 by subcloning of pT181 Taq I fragments to a derivative vector as shown in Fig. 5 (7).

Copy mutants

Evidence implicating the above-mentioned inhibitor in pT181 replication control was provided by the isolation and analysis of copy mutants (8). These were obtained by selection for increased resistance to tetracycline and their plasmid linkage was verified by outcrossing. A screening gel showing a set of typical copy mutants is presented in Fig. 6. Samples are sheared, whole-cell minilysates (9) that are loaded in carefully calibrated amounts; gels of this type are scanned by fluorescence densitometry to determine copy numbers directly (10).

The copy mutants could be divided into 2 classes on the basis of resistance or sensitivity to CopA inhibition. Resistant mutants are referred to as dominant, sensitive as recessive. Included among the copy mutants are 3 short spontaneous deletions, 144, 256 and 257. All 3 are dominant and map in the copA region. One of them, Δ144, was cloned to pE194 and shown not to express CopA activity, indicating that the determinants of the inhibitor and its target are very closely linked and probably overlapping (8). Sequence analysis

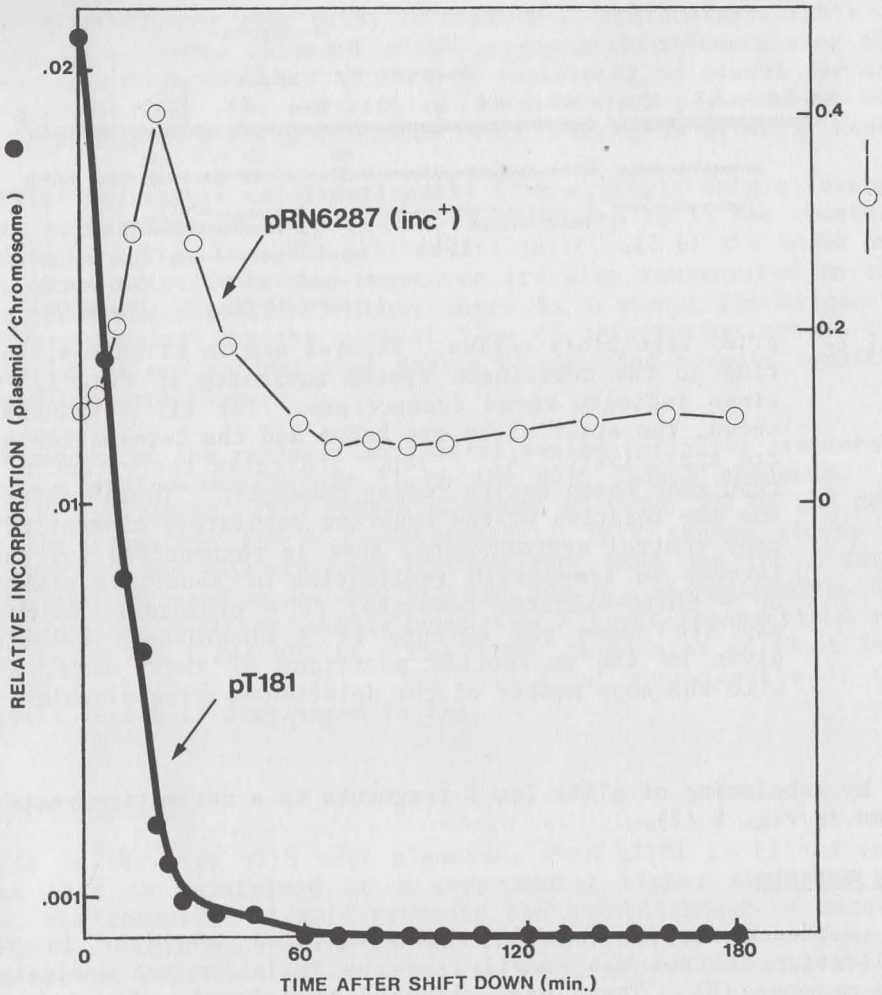


Fig. 3. Inhibition of the replication of pT181 by copA. pRN6287 is a derivative of pE194 (Tsr) to which has been cloned the Taq I-A fragment of pT181, indicated by "(inc$^+$)". Replication of pT181 (solid circles) and pRN6287 (open circles) was monitored following a temperature shift from 42°C to 32°C by successive 5 min pulses with ^{3}H thymidine. Lysates were prepared and separated by agarose gel electrophoresis and the resulting bands were excised and counted. The plasmid counts were normalized by dividing by the counts in the corresponding chromosomal bands. Note the difference in scale for the 2 plasmids.

showed that all the copy mutants are located in the copA region. The recessive copy mutants synthesized RNA I and RNA II either in reduced amounts or in abnormal patterns--thus cop-619 and 609 syn-

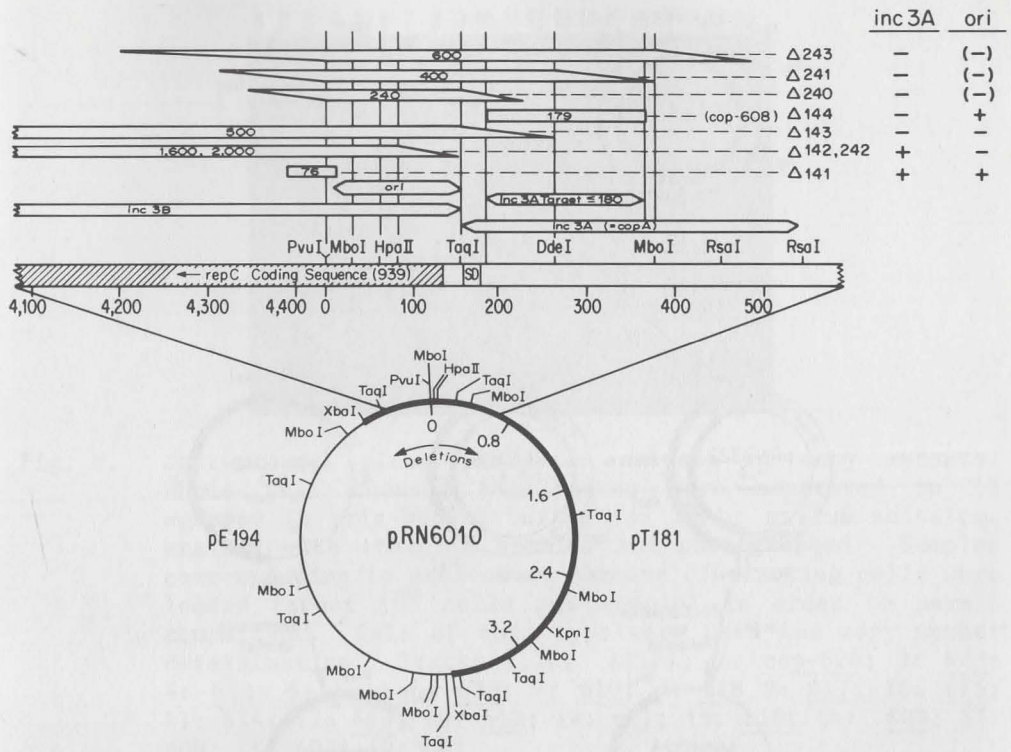


Fig. 4. Deletion map of the repC control region. Deletions 141-143 and 240-243 were generated by enzymatic digestion of Pvu I-linearized pRN6010 (see Fig. 5) DNA and were mapped by restriction analysis and scored for Inc3A activity (see text) and origin activity (5). Numbers indicate estimated sizes of the deletions and tapering portions indicate the limits of uncertainty of the locations of the ends. The locations of Δ141 and Δ144 have been determined precisely by sequencing (2,4). The locations of inc3A and inc3B were defined by cloning (see text). The Inc3A target was defined by Δ144.

thesized much less of the 2 than the wild-type; cop-621, 622, and 629 synthesized only RNA I or RNA II but not both (see Fig. 7). The dominant mutants, on the other hand, always synthesized greatly elevated amounts of these 2 RNA species. These results indicate that the inhibitory activity expressed by the copA gene is due to the countertranscripts and that both may be required for normal copy control.

Regulatory Levels

Preliminary measurements of the relative countertranscript

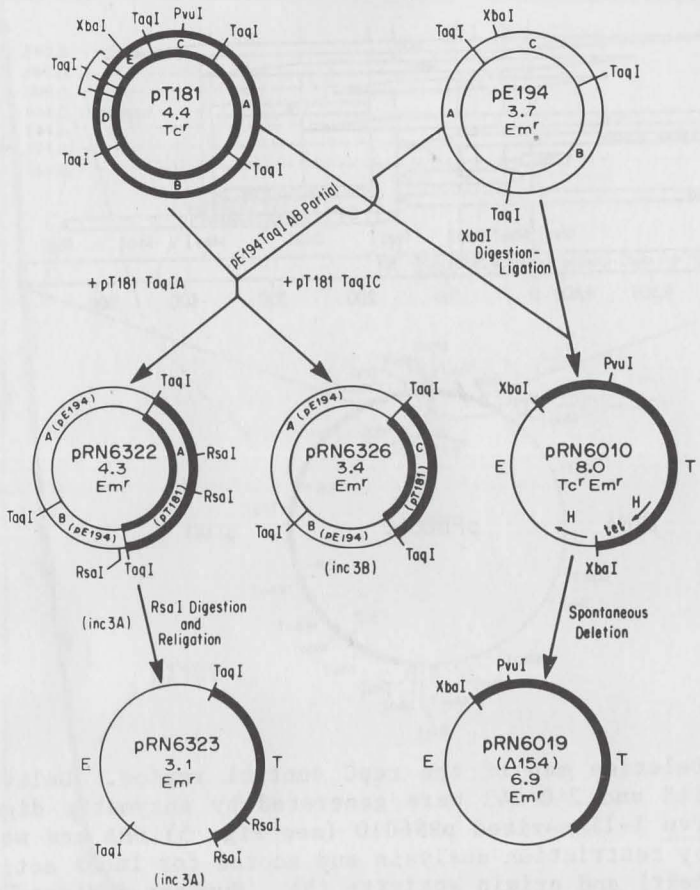


Fig. 5. Plasmid constructions. Only restriction sites involved in the cloning are shown. pT181 and its fragments are shown with heavy lines; pE194 and its fragments are drawn lightly. At right is shown the construction of pRN6010 by Xba I digestion and ligation. pRN6019 arose by the spontaneous deletion of 2 kb between the arrows marked "H". At left is shown the cloning inc3A (copA) and inc3B. For inc3A, the largest Taq I fragment (A) of pT181 was ligated to a partial Taq I digest of pE194 and the resulting chimera was digested with Rsa I and re-ligated. The final subclone, pRN6323, lacks the Rsa I fragment between position 570 of pT181 and 611 of pE194 and has therefore lost a 23-bp stretch of pE194 DNA between the Rsa I (611) and Taq I (588) sites (22) at 6 o'clock as shown. For inc3B, the third largest Taq I fragment of pT181 (C) was ligated to the same partial digest of pE194 (pRN6326). Both of these clones lack the non-essential Taq I-C fragment of pE194.

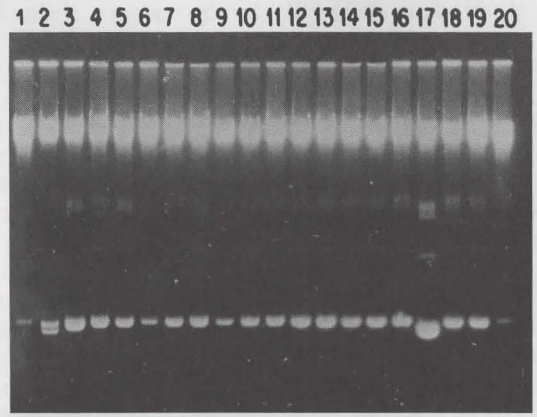


Fig. 6. Agarose gel electrophoretic analysis of copy mutants. Whole cell sheared minilysates were separated on 1% agarose in tris-borate buffer for 18 hr at 2.5 volts/cm, stained with ethidium bromide and photographed. Samples corresponding to equivalent numbers of starting cells were loaded (about 10^8 cells per sample) in order to permit comparison. Gels of this type were used for copy number determination. Tracks 1,20: pT181; 2: cop-626; 3: 623; 4: 622; 5: 621; 6: 620; 7: 619; 8: 618 9: 617; 10: 615; 11: 614; 12: 613; 13: 612; 14: 611; 15: 610; 16: 609; 17: 608; 18: 607; 19: 601.

The heavy upper and lower bands represent sheared chromosomal and supercoiled plasmid monomeric DNA, respectively. The uppermost band represents a small amount of material that remains in the sample well. The intermediate bands represent plasmid isomers including oligomers and relaxed monomers.

levels in copy mutants suggests that copy number is determined by the countertranscript concentrations per se. For example, in promoter-down mutants such as cop-619, the countertranscript levels are very close to wild-type despite a 10-fold increase in copy number; in other words, a 10-fold increase in countertranscript gene dosage is required to establish the standard regulatory inhibitor level. On the other hand, with dominant mutants, the countertranscript levels are increased in proportion to the copy number; because the mutant plasmid is less sensitive to the inhibitor, a higher concentration of the latter is required to achieve a regulatory level. Note that the target concentration seems not to be important.

Analysis of the repC mRNA Leader Sequence

A possible mechanism by which such countertranscripts might

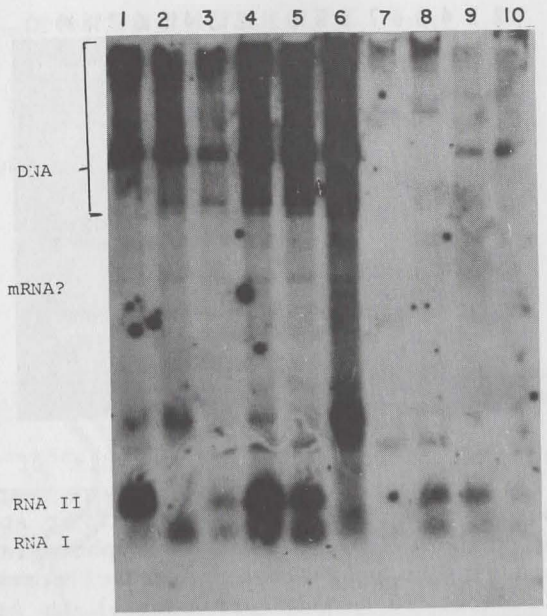
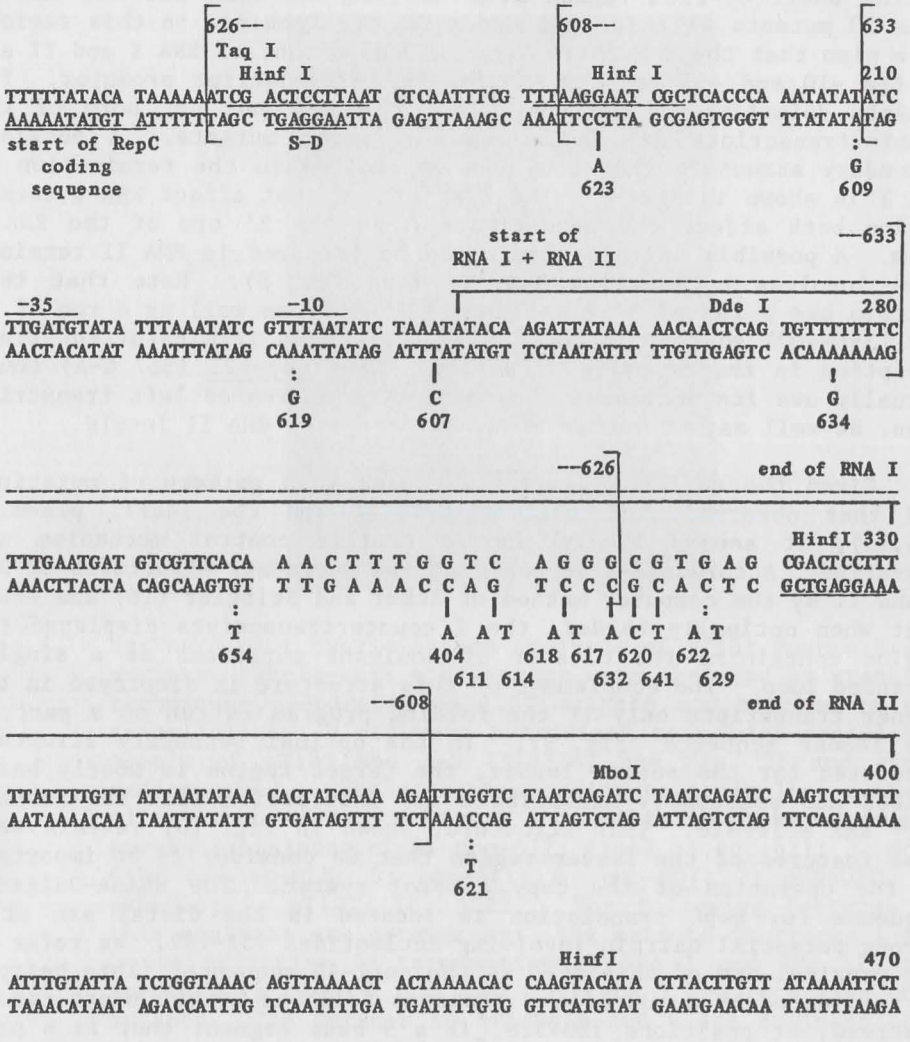


Fig. 7. Northern blot hybridization analysis of pT181 countertranscripts. Whole-cell minilysates were prepared by standard techniques, separated on 1.5% agarose and blotted to nitrocellulose. Blots were hybridized to a nick-translated sample of pT181 Mbo I-D (31-376; see Fig. 2) and autoradiographed. Samples corresponding to equivalent numbers of cells were run. In track 1 is a lysate prepared from a strain containing pT181 cop-622; track 2: cop-621; 3: cop-619; 4: cop-618; 5: cop-611; 6: cop-607; 7: pRN6050; 8: pRN6010; 9: pT181; 10: pC221. The following features of this analysis are noteworthy: the mutants in tracks 1 and 2 are defective in termination of RNA I and RNA II, respectively; these 2 and that in track 3 are recessive copy mutants. The mutants in tracks 5 and 6 are dominant copy mutants and, typically, overproduce the countertranscripts. The mutant in track 6 is also dominant but is evidently defective in countertranscript production as well as sensitivity. The extracts in tracks 7 and 8 are from strains containing the 2 orientations of the pT181::pE194 cointegrate; the difference in countertranscript levels suggests the possibility of transcriptional interference in 1 of the 2 orientations. Finally, the extract in track 10 is from a strain containing pC221, a plasmid that is compatible with but related to pT181.

regulate pT181 replication was suggested by analysis of the sequence of the repC mRNA leader region and of the copy mutations as shown in Fig. 8. Note that there is a strong cluster of dominant mutations

in the short GC-rich region from 308-320 and that all but one of some 20 mutants of this type had a GC to AT change in this region. Note also that the 2 mutants with reduced amount of RNA I and II are in the -10 and -35 regions of the countertranscript promoter. The mutants defective in the synthesis of either but not both of the countertranscripts are evidently terminator mutants. A possible secondary structure that could be implicated in the termination of RNA I is shown in Fig. 9. The 2 mutations that affect RNA I termination both affect the penultimate G in the 3' arm of the RNA I stem. A possible hairpin that could be involved in RNA II termination involves nucleotides 358-399 (see Fig. 8). Note that this hairpin has a run of 5' A residues (358-361) as well as a run of 3' U's (396-400) so that it could also serve as a terminator for transcription in the opposite direction. Thus cop-621 (367 G-A) could actually owe its increased copy number to increased left transcription, as well as, or rather than, to decreased RNA II levels.

Given the striking parallel between this pattern of mutations and that observed for ColE1 (11,12,13) and the IncFII plasmids (14,15), it seemed likely that a similar control mechanism was operative. Accordingly, we analyzed the secondary structures of RNA I and II by the computer method of Zuker and Stiegler (16) and found that when optimally folded, the 2 countertranscripts displayed the region containing the cluster of dominant mutations as a single-stranded loop. The complement of this structure is displayed in the leader transcripts only if the folding program is run on a part of the leader sequence (Fig. 9). In the optimal secondary structure predicted for the entire leader, the target region is mostly base-paired and presumably unavailable for interaction with the inhibitory RNA molecule. This structure, shown in Fig. 10, reveals several features of the leader region that we consider to be important in the operation of the copy control system. The Shine-Dalgarno sequence for RepC translation is located in the distal arm of a strong potential hairpin involving nucleotides 151-192. We refer to the proximal arm of this dyad as the anti-SD sequence. This hairpin is terminated by a run of 6 U residues followed by the start codon. Upstream, at positions 320-328, is a 9 base segment that is a precise direct repeat of the Shine-Dalgarno sequence and flanking bases. In the computer-generated secondary structure of the leader, the upstream segment is paired with the anti-SD segment which would pre-empt the formation of the downstream hairpin, leaving the SD segment single-stranded and in a configuration that would be accessible for translation. This is referred to as the active configuration. We propose that the early configuration of the leader is the actual target for RNA I/II, that the interaction would involve the target loop as a nucleation site, and that an RNA-RNA duplex would form, as is the case with ColE1 (17) and, presumably, with the IncFII plasmids. The consequence of the RNA-RNA interaction would be the formation of the downstream stem in which the SD sequence is fully base paired and unavailable for the initiation of RepC translation. Additionally, this hairpin could serve as an early termina-



| = Dominant copy mutant (resistant to Inc3a, i.e. trans-acting, wild-type small RNAs).

: = Recessive copy mutant (sensitive to Inc3a).

Fig. 8. Sequence of the pT181 copy control region between bases 141 and 470. Restriction enzyme recognition sites are indicated as well as a putative ribosome binding site and start site for the RepC coding sequence; read from right to left. Base sequence changes in several independently isolated copy mutants of pT181 are as follows:

Fig. 8 (Continued)

| = Dominant mutation

: = Recessive

! = Temperature sensitive for replication

tion site (attenuator) for repC mRNA transcripts. We refer to this as the inactive configuration. The model is thus a dynamic one in which the critical inhibitory interaction occurs before transcription of the leader has progressed beyond nucleotide 140. Any leader transcripts that progress a further 10 bases are predicted to refold into the active configuration and to be insensitive to the inhibitory interaction. Any transcripts that are "caught" by RNA I/II before refolding would be irreversibly inactivated. The level of RNA I/II, therefore, would determine the probability that any transcript would progress to an active form and by this means would set the rate of RepC production and therefore the replication rate. Note that this process is regarded as a steady state system rather than one in which fluctuations in the inhibitor concentration have an all-or- none effect on replication as was suggested in the original formulation of the inhibitor-dilution model (18). Available evidence in support of this model is mostly circumstantial and is as follows:

1. We have replaced the G at position 168 (see Fig. 8) with a C by synthetic oligonucleotide-directed mutagenesis and found that the mutant sequence is translationally inactive in E. coli. This supports the identification of the SD sequence as indicated and therefore of the RepC start (J. Kornblum et al., unpub. data).

2. A spontaneous mutation (cop-623) at position 189 (C to A) has a dominant high-copy phenotype and is completely indifferent to RNA I/II inhibition. This mutation changes the calculated free energy of the SD-anti SD loop from -14 to -6 kcal, supporting the conclusion that the anti-SD segment is critical for the regulatory interaction.

3. Sequence analysis of pC221, a closely related S. aureus plasmid that has a high degree of homology with pT181 in the leader region, has revealed a hairpin similar to that seen in pT181 positions 151-192, as well as a direct repeat of the putative SD sequence upstream (unpub. data). As shown in Fig. 11, however, the pC221 anti-SD sequence differs at 2 positions from the corresponding pT181 sequence and these 2 differences are mirrored by compensating differences in the putative pre-emptor sequence.

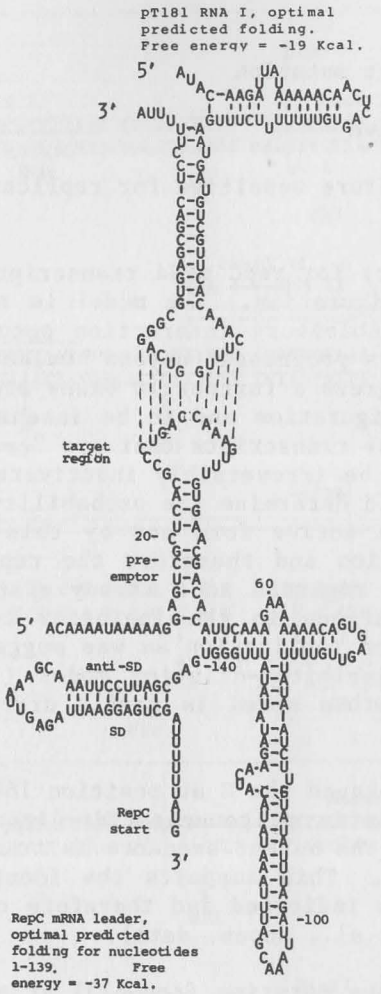


Fig. 9. Possible interaction between computer-predicted secondary structures for RNA I and the repC leader. In this model, the folding program has been run only on the first 140 nucleotides of the leader, as indicated. The secondary structure from 140 to the 3' end was predicted independently. Note that the target region is displayed as a single-stranded loop and that the Shine-Dalgarno sequence is fully base-paired.

4. Deletion of nucleotides 208-280 results in a high copy-number phenotype in which the mutant plasmid is insensitive to CopA inhibition. A T to C mutation at position

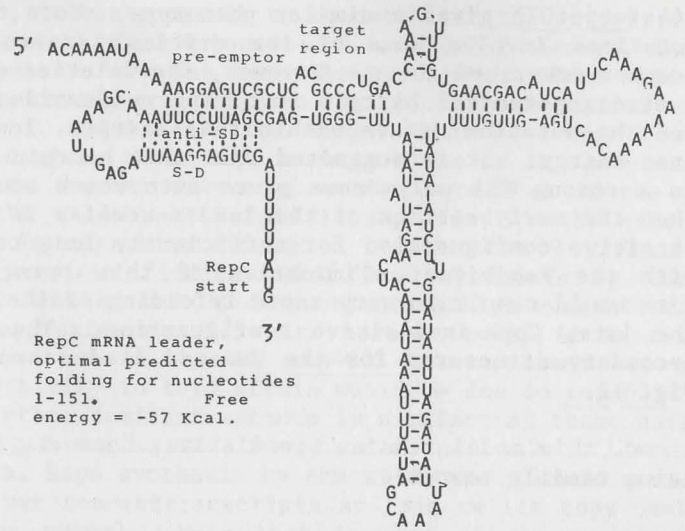


Fig. 10. Predicted secondary structure for the repC mRNA leader. (See text).

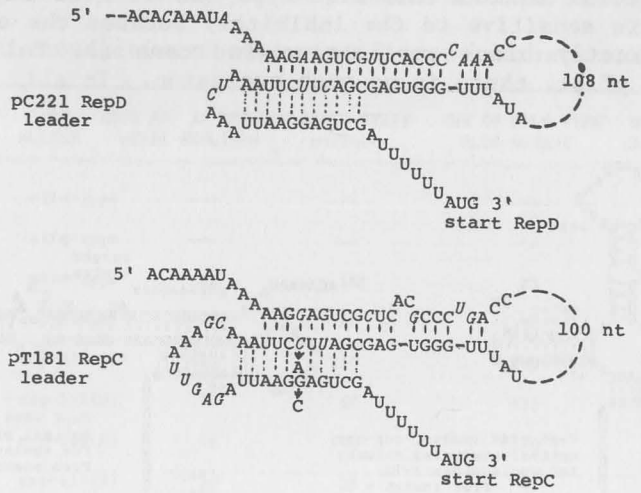


Fig. 11. Partial representation of the repC mRNA leader secondary structure for pT181 compared to the corresponding region of pC221. Note that the 5' end of the putative repD leader is uncertain. The nucleotides in the 2 corresponding regions of the 2 plasmids that do not match are shown in italics.

244 (cop-607) gives a similar phenotype. Note that these mutations involve none of the critical interacting sequences described above. However, the deletion eliminates a strong potential hairpin involving nucleotides 203-258, and the mutation occurs within this hairpin, lowering its free energy. It is suggested that this hairpin may serve as a strong RNA polymerase pause site which would ensure that the early section of the leader remains in the CopA-sensitive configuration for sufficiently long to interact with the inhibitor; elimination of this putative pause site would result in very rapid refolding of the leader to the late, CopA-insensitive configuration. The predicted secondary structures for the deleted leader are shown in Fig. 12.

At present, this model remains speculative; however, it has the virtue of being readily testable.

INDIRECT REGULATION

Non-exclusion

For ColE1, copy mutants that produce wild-type RNA I but are insensitive to it exclude the wild-type (as well as any other mutants that are sensitive to the inhibitor) because the elevated RNA I levels directly block replication in trans (B. Polisky, pers. comm.). For pT181, there is no such exclusion. In all cases thus

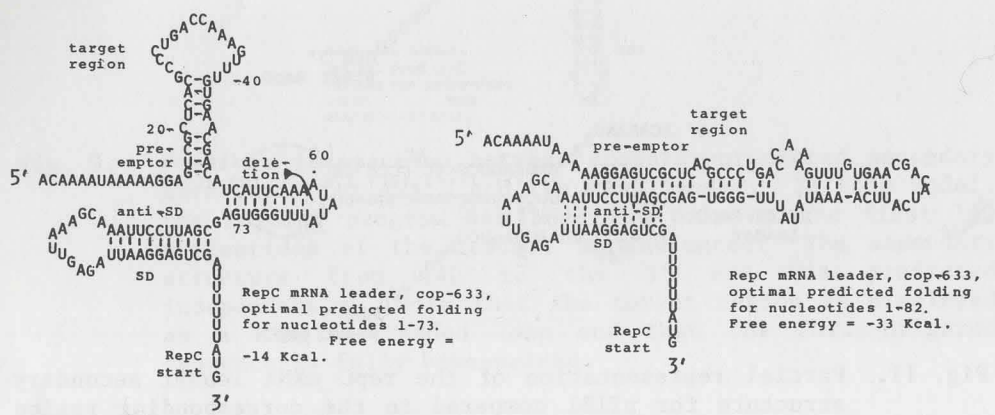


Fig. 12. Predicted secondary structures for the partially and completely folded cop-633 repC mRNA leader. Nucleotides are numbered from the 5' end of the leader in this diagram and the numbers do not correspond to the primary nucleotide numbers as shown in Fig. 8.

far tested, it was possible to co-establish the wild-type plasmid or any of the recessive mutants with any other mutant, including one dominant mutant that has been shown to overproduce wild-type RNA I and RNA II, namely cop-623 (see Fig. 8). These results are presented in Tab. 1, which lists the mutants that have been tested in this manner, and gives their copy numbers alone or in the presence of the wild-type or another mutant plasmid. Note that in the presence of cop-623, the wild-type plasmid is amplified to the extent that the copy pool is approximately split between the 2 plasmids. On the basis of previous findings that RepC synthesis by the wild-type plasmid is blocked when the countertranscript concentration is elevated to approximately 2.5x its normal level, it is inferred that the same is true in the WT/ cop-623 heteroplasmid, so that all of the RepC activity in this strain would be due to cop-623. The situation for other dominant mutants is similar; as these have mutations in the target region and therefore do not make wild-type countertranscripts, RepC synthesis by the wild-type plasmid would be blocked by its own countertranscripts so long as its copy number is elevated above normal. Note that in most of the examples, the copy pool is approximately split, as would be expected if the constituent plasmids can compete equally for all of the RepC protein present. In a few cases, the copy pools are asymmetric; the plasmid with the higher copy number evidently has a competitive advantage over the other; the basis of such advantage is not presently understood. An additional feature of these heteroplasmids is that one can predict

Tab. 1. Interactions between pT181 copy mutants and pSA5000.

PLASMID	COP ALLELE	COPY NO. IN HETEROPLASMID STATE pT181 REPLICON	pSA5000	SUM OF WILD-TYPE PLUS MUTANT	HOMOPLASMID COPY NUMBER
pT181	wild-type	---	---	---	22
pSA5000	wild-type	---	---	---	22
pT181	wild-type	12	10	22	22
pRN8009	cop-609(R)*	22	32	52	74
pRN8011	cop-611(D)*	25	110	140	200
pRN8020	cop-615(D)	70	63	130	150
pRN8023	cop-618(D)	46	170	210	210
pRN8024	cop-619(R)	20	21	41	140
pRN8026	cop-621(R)	85	23	110	150
pRN8027	cop-622(R)	32	22	54	120
pRN8061	cop-623(D)	110	130	240	400

*R = recessive; D = dominant or overlap mutant.

the approximate total pools on the basis of the type of mutant plasmid that is present. Thus, for recessive mutants, the pool is approximately twice the size of that for the wild-type alone; here, both plasmids can synthesize RepC but only one can synthesize active inhibitor. In order to achieve the regulatory inhibitor level, the inhibitor-synthesizing plasmid must reach its normal (wild-type) copy number. Since the inhibitor-negative plasmid can compete equally for RepC, it also reaches the same level; hence, the double-sized pool. For dominant mutants, only the mutant can synthesize RepC but both plasmids utilize it; thus, the overall pool should be somewhat less than that of the mutant alone, which is roughly true in most cases.

Incompatibility

Although indirectly regulated plasmids do not show exclusion in tests such as those just described, they do show segregational incompatibility. The segregation rates for heteroplasmids containing isogenic replicons can be predicted on the basis of copy numbers according to a Markov Chain analysis for random replication plus random assortment with equipartitioning (19). In practice, determination of segregation rates for heteroplasmids with high copy numbers is inconvenient and we have adopted a different strategy for evaluating incompatibility. This strategy involves a phenomenon referred to as reciprocal intrapool variation (20), in which selection for one of the plasmids in a heteroplasmid pool causes amplification of that plasmid at the expense of the other if the 2 are segregationally incompatible but not if they are compatible. A typical example for wild-type incompatible (Inc3) plasmids pT181 and pSA5000, and for compatible plasmids pT181 and pC194 is shown in Fig. 13 and 14. Note in particular that the reversal of relative copy numbers is virtually complete before there is any detectable segregation for the incompatible pair and that there is no detectable variation for the compatible pair. Note also that the overall copy pools remain constant in both cases. Similar results are observed for heteroplasmid strains containing wild-type and mutant or pairs of mutant plasmids, except that where only one of the plasmids can synthesize RepC, the overall pool does not remain constant. Selection for the RepC-producing plasmid elevates the overall pool and selection for the other lowers the overall pool, very much as one would expect (20).

Inhibitor-target specificity and incompatibility

As has been shown for ColE1 (13) and for several IncFII plasmids (21), mutations affecting the specificity of inhibitor-target recognition for directly regulated plasmids have profound effects on segregational incompatibility; if the mutant inhibitor is less active against the target of another mutant or the wild-type, then the strength of the observed segregational incompatibility is decreased

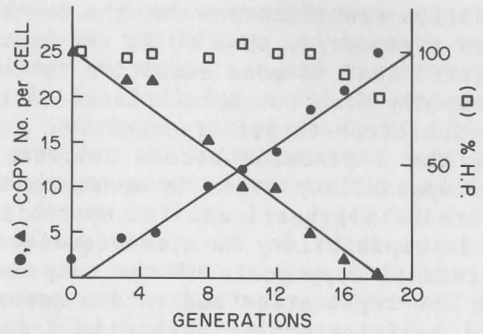


Fig. 13. Reciprocal intrapool variation for isogenic wild-type plasmid replicons. A culture of a strain containing pT181 and pSA5000 was grown exponentially for 20 generations in CY broth containing Cm and Tc at 20 and 1 μg/ml, respectively, then shifted into CY broth containing Cm and Tc at 1 and 10 μg/ml, respectively, and followed for a further 18 generations of exponential growth. The culture was sampled periodically and analyzed by gel electrophoresis for the relative amounts of the 2 plasmids and simultaneously plated to score for the heteroplasmid population. Solid triangles: copy number of pSA5000; solid circles: copy number of pT181; open squares: percentage of heteroplasmids.

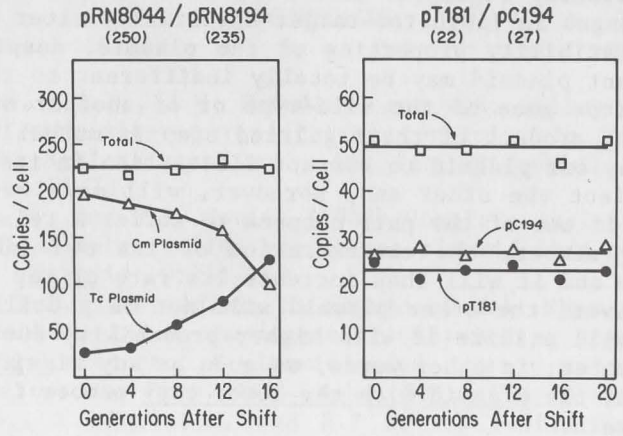


Fig. 14. Effect of antibiotic selection on heteroplasmids containing pT181 copy mutants (left) and compatible plasmids (right). Experiments performed as in Fig. 13. The 2 mutants whose intrapool data are shown at left have non-cross-reacting inhibitors and targets. The compatible plasmids used in the experiment shown at right are pT181 and pC194. The cultures were assayed periodically for plasmid content and for heteroplasmid frequency.

(13); if the recognition specificities for the contralateral inhibitors and targets are asymmetric, then so is the segregational incompatibility; the plasmid that is more sensitive to the other's inhibitor is lost more rapidly from the heteroplasmid strain. In extreme cases, where the inhibitor-target recognition specificities are changed completely, the 2 plasmids become compatible. This establishment of new incompatibility types is understandable in terms of the stochastic nature of segregational incompatibility; in particular, segregational incompatibility is a consequence of random fluctuations in the individual copy pools of the component plasmids, due to random selection for replication and random assortment for partitioning. Since the self-corrective mechanism does not distinguish the component plasmids, it cannot correct the disproportions and the eventual result is segregation at rates that are predictably determined by the overall copy number of the heteroplasmid pool. The mutations that change inhibitor-target recognition serve to establish independent self-correcting copy pools for any 2 or more such plasmids in the heteroplasmid state; this has the effect of making the 2 plasmids segregationally compatible. It is also obvious that in such cases, if one were to clone the inhibitor determinant to an independent replicon, it would have an effect on other plasmids in trans that was a reflection of its target recognition specificity. This is amply demonstrated by the experiments of Lacatena and Cesareni (11,12) with ColE1 mutants; conversely, it is predicted that mutants selected for insensitivity to the wild-type (or mutant) inhibitor would show the same type of incompatibility changes.

For indirectly regulated plasmids, it is predicted that in no case will changes in inhibitor-target specificity alter the segregational incompatibility properties of the plasmid, despite the fact that the mutant plasmid may be totally indifferent to the passively cloned inhibitor gene of the wild-type or of another mutant. This is because the product of the regulated step is mutually available; any attempt by one plasmid to correct fluctuation in its copy number will also affect the other and, moreover, will make matters worse. For example, if one of the pair happens to suffer a relative diminution of copy number, the concentration of its own inhibitor will also decrease and it will then increase its rate of rep protein synthesis. However, the other plasmid will not only utilize this new product, it will utilize it with higher probability due to its elevated copy number; in other words, as soon as any significant fluctuation occurs, the plasmid with the lower copy number is doomed to a stochastic death.

This prediction has been verified with pT181. The inhibitor region (nucleotides 158-570) has been cloned to pE194 for several of the dominant pT181 copy mutants and the cloned inhibitor has been found to have lower activity than the wild-type inhibitor towards either wild-type or other dominant mutants. In some combination, no inhibitory activity could be demonstrated, the target plasmid having the same copy number in the presence as in the absence of the cloned

inhibitor determinant. Heteroplasmids were constructed for several pairs of plasmids whose inhibitors and targets were mutually indifferent to one another by this test and these were tested for segregational incompatibility both by growth in non-selective medium and by the reciprocal intrapool variation test. Because of the relatively high copy numbers of these mutants, segregational incompatibility was difficult to measure accurately, although it was always demonstrable. As shown in Fig. 14, reciprocal intrapool variation was readily demonstrable for one of these pairs. The rate of reversal of the pool was somewhat slower than that for the wild-type pair; this difference is tentatively attributed to the larger size of the mutant copy pool; experiments to test this by means of isogenic plasmids with the same copy pool are in progress.

ACKNOWLEDGMENTS

This work was supported by research grants from the American Cancer Society (MV-8D) and National Institutes of Health (GM14372) to R.P.N. S.J.P. held a National Research Service Award from the National Institutes of Health (F32-GM07899); C.C.K. was a postdoctoral fellow of the Leukemia Society of America, Inc.; S.C., A.G., and S.K.H. were trainees in the Graduate Assistants Program of New York University Medical School. Expert technical and secretarial assistance of Soraya Moghazeh and Paula Offricht, respectively, are gratefully acknowledged.

REFERENCES

1. Grindley, N.D.F., J.N. Grindley, and W.S. Kelley (1978) Mutant plasmid with altered replication control. In Microbiology - 1978, D. Schlessinger, ed. Am. Soc. Microbiol., Washington, D.C., pp. 71-73.
2. Khan, S.A., and R.P. Novick (1983) Complete nucleotide sequence of pT181, a tetracycline resistance plasmid from staphylococcus aureus. Plasmid 10:251-259.
3. Novick, R.P., G.K. Adler, S. Majumder, S.A. Khan, S. Carleton, W. Rosenblum, and S. Iordanescu (1982) Coding sequence for the pT181 repC product: A plasmid-coded protein uniquely required for replication, Proc. Natl. Acad. Sci., USA 79:4108-4112.
4. Khan, S., S. Carleton, and R.P Novick (1981) Replication of plasmid pT181 DNA in vitro: Requirement for a plasmid coded product. Proc. Natl. Acad. Sci., USA 78:4902-4906.
5. Khan, S.A., G.K. Adler, and R.P. Novick (1982) Functional origin of replication of pT181 plasmid DNA is contained within a 168-base-pair segment. Proc. Natl. Acad. Sci., USA 79:4580-4584.
6. Kumar, C., and R.P. Novick (1984) Plasmid pT181 replication is regulated by two countertranscripts. Submitted to Proc. Natl. Acad. Sci., USA.

. Novick, R.P., G.K. Adler, S.J. Projan, S. Carleton, S.K. Highlander, A. Gruss, S.A. Khan, and S. Iordanescu (1984) Control of pT181 Replication I. The pT181 copy control function acts by inhibiting the synthesis of a replication protein. Submitted to Embo J.
. Carleton S., S.J. Projan, S.K. Highland, S.M. Moghazeh, and R.P. Novick (1984) Control of pT181 Replication II. Mutational analysis. Submitted to Embo J.

. Eckhardt, T. (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584-588.
. Projan, S.J., S. Carleton, and R.P. Novick (1983) Determination of plasmid copy number by fluorescence densitometry. Plasmid 9:182-190.
. Lacatena, R.M., and G. Cesareni (1981) Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication. Nature 294:623-626.
2. Lacatena, R.M., and G. Cesareni (1983) Anatomy of the target of the inhibitor of ColE1 replication. In Abstracts of the 1982 EMBO Conference, "Replication of Prokaryotic DNA," The Netherlands, pp.62-65.
3. Tomizawa, J.-I., and T. Itoh (1981) Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. Proc. Natl. Acad. Sci., USA 78:6096-6100.
4. Brady, G., J. Frey, H. Danbara, and K. Timmis (1983) Replication control mutations of plasmid R6-5 and their consequences for interactions of the RNA-I control element with its target. J. Bact. 154:429-436.
5. Nordstrom, K., S. Molin, and H. Aagaard-Hansen (1981) Plasmid R1 incompatibility. In Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, R.C. Clowes, S.B. Levy, and E.L. Koening, eds. Plenum Press, New York, pp.291-301.
6. Zuker, M., and P. Stiegler (1981) Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. 9:133-148.
7. Tomizawa, J.-I., and T. Itoh (1982) The importance of RNA secondary structure in ColE1 primer formation. Cell 31:575-583.
8. Pritchard, R.H., P.B. Barth, and J. Collins (1969) Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microb. 19:263-297.
9. Novick, R.P., and F. Hoppensteadt (1978) On plasmid incompatibility. Plasmid 1:421-434.
0. Projan, S.J., and R.P. Novick (1984) Reciprocal intrapool variation in plasmid copy numbers: A characteristic of segregational incompatibility. Plasmid (in press).
1. Easton, A.M., and R.H. Rownd (1982) The incompatibility product of IncFII R plasmid NR1 controls gene expression in the plasmid replication region. J. Bact. 152:829-839.
2. Horinouchi, S., and B. Weisblum (1982) Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, linosamide and streptogramin type B antibiotics. J. Bact. 150:815-825.

CONSTRUCTION OF ColE1 RNA1 MUTANTS AND ANALYSIS OF THEIR FUNCTION IN VIVO

Barry Polisky, Joseph Tamm, and Tim Fitzwater

Program in Molecular, Cellular, and Developmental Biology
Department of Biology
Indiana University
Bloomington, Indiana 47405

ABSTRACT

We have carried out experiments designed to investigate the relationship between structure and function for the ColE1 RNA1 species. RNA1 is a small RNA (108 nucleotides) that has been implicated in copy number control of the multicopy plasmid ColE1. In vitro, RNA1 inhibits the processing of the primer precursor required for initiation of DNA replication. The RNA1 gene is entirely complementary to the 5'-terminal region of the primer. We have functionally separated these 2 RNA species by cloning the RNA1 gene downstream from the S. marcesens trp promoter. When cloned in a ColE1-compatible plasmid, a trp-RNA1 fusion has been shown to mediate ColE1-type incompatibility in vivo. The construction scheme described here also generates mutant RNA1 species with altered sequences at the 5' terminus of RNA1 which have been assayed for function in vivo. These experiments have indicated that sequences at the 5' terminus play a critical role in RNA1 function.

INTRODUCTION

ColE1 is a small plasmid of Escherichia coli that has proven to be a valuable model for studies of DNA replication control. By biochemical and genetic dissection of the sequence and replication machinery requirements of this plasmid, it has been possible to isolate and characterize the major plasmid-encoded elements involved in the initiation and regulation of plasmid replication. ColE1 exists at about 20-30 copies per chromosome and is totally dependent on

host replication enzymes (1,2). Its replication does not require any plasmid-encoded information that must function in trans in a "positive" genetic sense (1,2). Indeed, a variety of genetic experiments involving copy-number mutants have indicated that the major mode of control was via the synthesis of plasmid-specific, negatively acting replication control elements (3,4).

The development of an in vitro replication system using supercoiled plasmid DNA and purified enzymes has revealed a detailed picture of the minimal requirements for high-fidelity replication initiation. The enzymatic components are RNA polymerase, DNA polymerase I and ribonuclease H (5). The ColE1 replication primer is initiated 555 nucleotides upstream from the origin by RNA polymerase and is endonucleolytically processed by RNase H to create a functional 3'-OH onto which DNA polymerase I adds deoxyribonucleotides (5).

It is obvious that ColE1 replication must be regulated at some level to account for its constant copy number and its genetic stability. A number of lines of evidence indicate that primer formation is the locus of control in vitro and that a central regulatory factor is a small RNA species encoded by ColE1 (6). This small RNA is transcribed from the DNA strand opposite to that encoding the primer and is entirely complementary to 5' terminal 108 nucleotides of the primer (7). Addition of purified ColE1 RNA1 to an in vitro ColE1 replication system results in inhibition of RNase H processing of the nascent primer and an inhibition of addition of deoxyribonucleotide incorporation by DNA polymerase I (6).

Like other plasmids, ColE1 displays incompatibility in vivo towards related replicons. One way to tie the in vivo incompatibility behavior with the in vitro replication results is provided by the incompatibility model proposed by Pritchard and colleagues (8,9). In its broadest sense this model suggests that incompatibility is a consequence of negative replication control mediated by trans-acting repressors which have specificity for replicons of a given incompatibility group. In conjunction with a random replication and/or segregation scheme this proposal can account for the incompatibility behavior of ColE1-type plasmids. In this model RNA1 is the molecular basis of incompatibility, acting in trans to lower the probability of DNA initiation as a consequence of its interaction with the replication primer (10,11).

We are interested in the structure and function of RNA1 for several reasons. First, we wanted to investigate whether RNA1 is sufficient to mediate ColE1 incompatibility in vivo. Second, RNA1 represents a novel type of repressor molecule in that its activity seemed likely to be mediated largely through its secondary and tertiary structural features and those of its target, the replication primer (10,11). Our long-term goal was to establish a genetic

system with which we could introduce mutations specifically in RNA1, test the effects of these changes in vivo on ColE1 replication, and then investigate the structural features of RNA1 that had been altered by these changes.

RESULTS AND DISCUSSION

Because of the physical overlap between RNA1 and the primer, point mutations in one create complementary changes in the other. We isolated a DNA fragment containing the RNA1 structural gene, but lacking functional elements of both the RNA1 promoter as well as primer promoter. This fragment, a 148 bp HaeIII-FNUdIII segment, was isolated from the cop$^+$ ColE1 plasmid pNOP42 (4). The scheme by which it was placed under the transcriptional control of the Serratia marcesens tryptophan promoter is depicted in Fig. 1a. The RNA1-containing fragment has blunt ends to which synthetic HindIII linkers were ligated. The resulting fragment was cloned into the unique HindIII site immediately downstream of the S. marcesens trp promoter present in the pBR322-derivative plasmid pM59. The resulting plasmid was designated pTF114. pTF114 also carries the pBR322-derived RNA1 gene and is ColE1-incompatible. To create a plasmid whose ColE1-incompatibility determinants were derived from the trp-RNA1 moiety, we replaced the pBR322 origin region with a 2055 bp PstI-SmaI fragment containing the replication origin of the ColE1-compatible plasmid pACYC177 (12). These manipulations created the plasmid designated pTF275 (Fig. 1a). To provide sufficient quantities of the trp repressor to control the multicopy trp operator we introduced a 1250 bp BamHI fragment that contains the E. coli trp repressor structural gene (13). This fragment was introduced into the unique BamHI site of pTF114. The HindIII downstream from the 3' end of the RNA1 gene in pTF275 was deleted after partial HindIII digestion and Bal31 treatment. These manipulations created a plasmid with a unique HindIII site located between the trp promoter and the ColE1 RNA1 gene. This plasmid was designated pTF325 (Fig. 1a).

Subsequent experimental steps were designed to create fusions of various types between the trp promoter and the RNA1 gene. Features of the DNA sequence relevant to these steps are shown in Fig. 1b. The overall strategy was to open the plasmid at the HindIII site and remove nucleotides from both termini with limited Bal31 nuclease digestion. Following ligation of the resulting ends the altered molecules were introduced into recipient E. coli by transformation. It should be noted that in pTF325 the ColE1 RNA1 gene transcript would be expected to contain an extra 49 residues at its 5' terminus if transcriptional initiation occurred at the trp start (see leftmost wavy line in Fig. 1b). In in vitro transcription experiments carried out with supercoiled PTF325 DNA and E. coli RNA polymerase a prominent band with the expected mobility was observed (results not shown). In addition, we have observed that

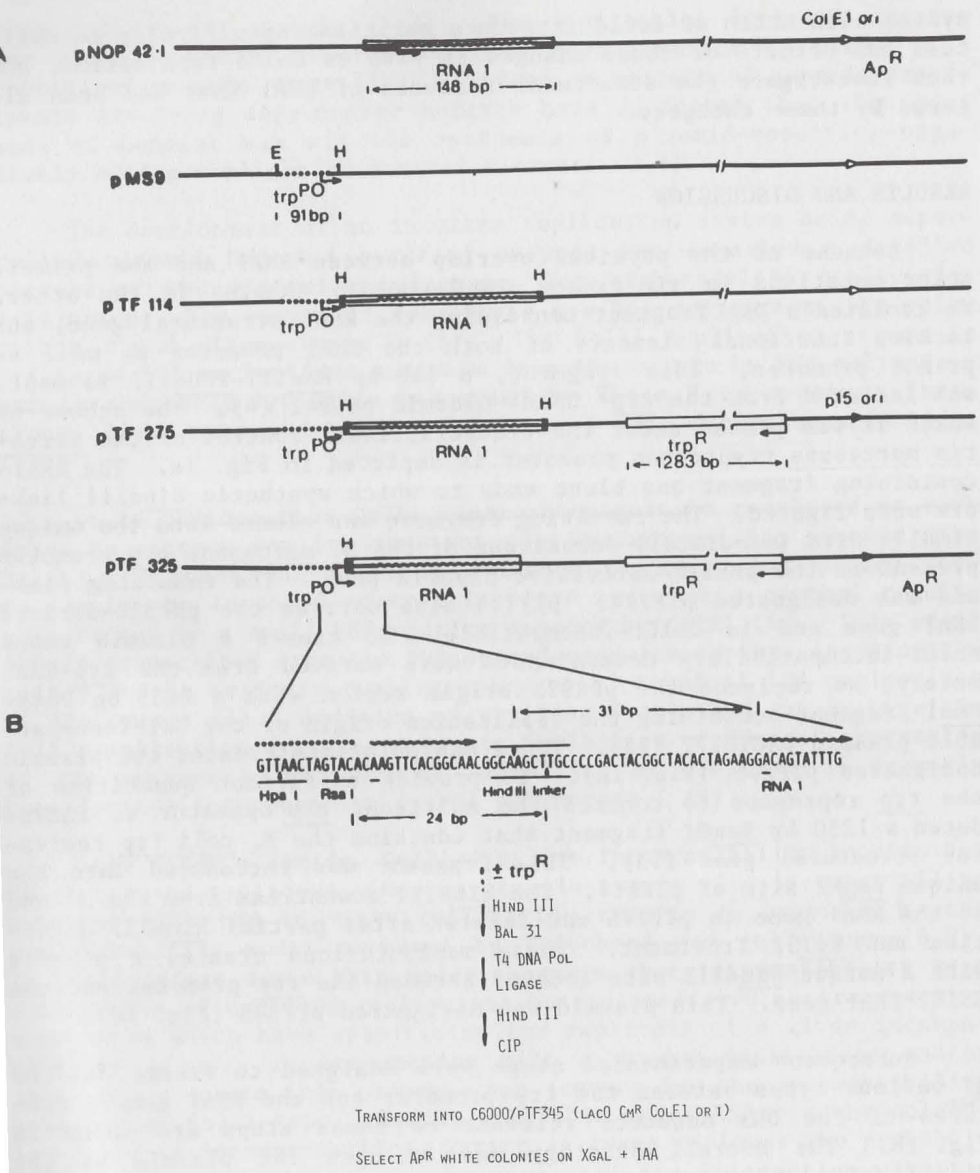


Fig. 1. (a) Scheme for the construction of trp promoter-RNA1 fusion plasmids; pNOP42°I is a 4.2x10^3 bp cop+ ColE1 plasmid. A 148 bp HaeIII-FnudII fragment was isolated from this plasmid by electroelution from a 6% (w/v) polyacrylamid gel. This fragment contains the coding region for RNA1 but lacks the RNA1 promoter and the replication primer promoter. Synthetic decameric HindIII linkers (BRL) were attached to this fragment which was then cloned into the unique HindIII site of pMS9. A plasmid with the proper orientation of insertion of the RNA1 fragment with re-

Fig. 1. (Continued)

spect to the trp promoter was selected and designated pTF114. pTF114 contains a ColE1-type replication origin, is incompatible with ColE1, and encodes Ap^R. A 2055 bp PstI-SmaI fragment containing the replication origin from the ColE1-compatible plasmid pACYC177 was inserted to replace the PstI-PruII fragment of pTF114 which contains the replication origin. The insertion re-creates the bla gene and the resulting plasmid encodes Ap^R. In addition, a 1250-bp BamHI fragment which encodes trpR from E. coli was isolated from ptrpR3 (13). This fragment was inserted into the unique BamHI site of pTF114. These manipulations resulted in pTF275 which was treated with HindIII under conditions designed to partially cleave at one of the 2 HindIII sites. One-cut linear molecules were isolated from a 1% (w/v) agarose gel and treated with 0.1 unit of Bal31 nuclease/µg DNA for 25 min at 30°C. Following extraction with phenol and precipitation with ethanol, 100 units of DNA ligase were added and the mixture was used to transform Ap^R E. coli C600 cells with selection for Ap^R. Transformats were analyzed for a plasmid that retained the trp promoter-proximal HindIII site but lacked the distal HindIII site. A plasmid designated pTF325 was isolated by this procedure. pTF325 is deleted for approximately 75 bp and lacks the distal HindIII site.

(b) The nucleotide sequence (hyphens omitted for clarity) of pTF325 in the region containing the trp operator, the unique HindIII site and the 5'-proximal nucleotides of ColE1 RNA1. The dots over the sequence designate sequences relevant for trp repressor binding. The underlined A residue adjacent to the RsaI site is the initiating nucleotide of the truncated trp leader sequence. The wavy line designates the start of RNA1 transcription and the initiating A residue of RNA1 is also underlined. The decameric HindIII site is shown with lines above and below the sequence. Arrows designate the sizes of particular regions or the direction of transcription.

Deletions were introduced into pTF325 as follows. 20 µg of pTF325 DNA were cut with HindIII (Biolabs) in TA buffer (33 mM-Tris-acetate, pH 7.9, 66 mM-potassium acetate, 10mM-magnesium acetate, 100 µg bovine serum albumin/ml, 0.5 mM-dithiothreitol) to completion, extracted with phenol, precipitated with ethanol, and resuspended at 1 µg/µl in TE buffer (10 mM-Tris·HCl, 1 mM-EDTA, pH 8). 1 µg portions in 25 µg 1 x Bal31 salts (200 mM-NaCl, 12.5 mM-$CaCl_2$, 12.5 mM-$MgSO_4$, 20 mM-Tris·HCl, pH 8.1, 1 mM-EDTA) were treated with 0.1 unit Bal31 (BRL) at 30°C for 25 min. Pilot experiments indicated that these conditions removed an average 30 bp from each end. After Bal31 treatment,

Fig. 1. (Continued)

portions were pooled, extracted with phenol, and precipitated with ethanol. The DNA was then treated with 2.5 units T4 DNA polymerase/μg (BRL) in TA buffer at 37°C for 5 min. 2 mM-dNTPs were added and repair proceeded for 60 min at 37°C, followed by 70°C heat-inactivation for 10 min. 4 μg of DNA were ligated with 100 units T4 DNA ligase in TA buffer plus 1 mM-ATP at 14°C overnight, and heat-inactivated at 70°C for 10 min, followed by HindIII digestion at 37°C for 2 hr to eliminate any residual supercoiled DNA. DNA was then extracted with phenol precipitated with ethanol, and resuspended in 50 μl of CIP buffer (50 mM-Tris·HCl, pH 9.0, 1 mM-$MgCl_2$, 0.1 mM-$ZnCl_2$, 1 mM-spermidine) and treated with 1 unit calf intestinal phosphatase (CIP; Boehringer Mannheim) at 37°C for 30 min. to prevent in vivo ligation of HindIII ends that had escaped Bal31 digestion. An additional unit of CIP was then added and incubated as before. CIP was inactivated by the addition of 40 μl water, 10 μl 10 x TNE buffer (100 mM-Tris·HCl, pH 8.0, 1m-NaCl, 10 mM-EDTA), and 5 μl of 10% (w/v) sodium dodecyl sulfate followed by incubation at 68°C for 15 min. DNA was then extracted with phenol and precipitated with ethanol prior to resuspension in TE buffer (pH 8.0) for transformation. IAA, indoleacrylic acid.

pTF325 is compatible with ColE1-derived plasmids in vivo under conditions in which trp-directed transcription is expected to be constitutive. These results indicated that the ColE1 RNA1 produced by pTF325 was not capable of mediating ColE1-type incompatibility in vivo, most plausibly as a consequence of structural alterations in the fusion RNA1. Consequently, it was possible to select for functional trp-RNA1 fusions by introducing the Bal31-treated pTF325 molecules into an E. coli strain harboring a ColE1 plasmid and screening recipients for those which, upon subsequent growth, had lost the ColE1 resident. For this purpose we used a ColE1 plasmid designated pTF345. This plasmid confers chloramphenicol resistance (CM^R) and also carries a fragment containing the lac operator of E. coli. As a consequence of its multicopy nature, this plasmid titrates the endogenous lac repressor making the host constitutive for β-galactosidase expression (14). Consequently, colonies harboring pTF345 are blue on X-gal plates. We expected that a functional trp-RNA1 fusion would displace pTF345 resulting in a white Cm^S colony on X-gal plates. Indoleacrylic acid, an inducer of the trp operon (15), was also included to ensure that the trp promoter was active.

Using this scheme we isolated a fusion derivative designated pTF421 that had the expected properties. DNA sequence analysis across the fusion joint indicated that a 50 bp deletion occurred in pTF421. In vitro, this plasmid produces an RNA1 with mobility identical to that of cop^+ pNOP42. Finally, analysis of the 5'-terminal

sequences of pTF421-encoded ColE1 RNA1 indicated that it is identical to wild-type ColE1 RNA1 (results not shown). Subsequent transformation experiments revealed that pTF421 could displace resident ColE1 plasmids even in the presence of excess tryptophan, suggesting that RNA1 synthesis was constitutive. DNA sequence analysis demonstrated that the basis for this expression was the extent of the deletion on the trp side which resulted in a defective operator sequence (results not shown).

The characteristics of pTF421 demonstrated that the ColE1 RNA1 structural gene provides sufficient information to convert a ColE1-compatible plasmid into a ColE1-incompatible plasmid. The results indicate that RNA1 is an important incompatibility determinant in vivo but do not prove that it is the only ColE1-encoded component involved in this phenomenon.

In addition to pTF421 we have isolated other Bal31 fusion derivatives that are capable of displacing a resident ColE1 plasmid. Some of these plasmids displace ColE1 residents with apparently slower kinetics than pTF421. Two possible explanations could account for this behavior. The spacing between the trp promoter and the 5 'terminus of wild-type RNA1 might have been altered resulting in less efficient expression of a functional RNA1 species in vivo. Alternatively the Bal31 deletion step might have resulted in efficiently expressed altered RNA1 species that contained sequence changes at its 5' terminus such as 5' extensions, deletions, or fusions with upstream sequences.

As a qualitative measure of the trp promoter strength we have transcribed candidate plasmids in vitro with E. coli RNA polymerase and assessed the extent of the transcription of the trp-RNA1 species. For the plasmids described in this report we detected no differences between the deletion derivatives and the parent plasmid pTF325 (results not shown).

To obtain a quantitative measure of differences on ColE1-type incompatibility mediated by different deletion derivatives we carried out segregation tests in vivo. Over a period of 80 generations of growth in the absence of selection a wide variety of segregation kinetics were observed (Fig. 3). pTF421, encoding wild-type RNA1 segregated essentially completely from pTF345 within 8 generations. As noted previously, pTF325, in which 49 nucleotides are appended to the 5' terminus of RNA1, displays no incompatibility with pTF345. The sequence of several other derivatives are schematically depicted in Fig. 2b. pTF370 encodes an RNA1 with 5 additional residues at its 5' terminus and displaces pTF345 with slower kinetics than pTF421. pTF365 has 15 additional residues and does not detectably displace pTF345.

Two additional derivatives are worthy of mention. These were picked from a collection of colonies in which no segregation between the incoming Bal31 plasmid and the resident occurred. We examined

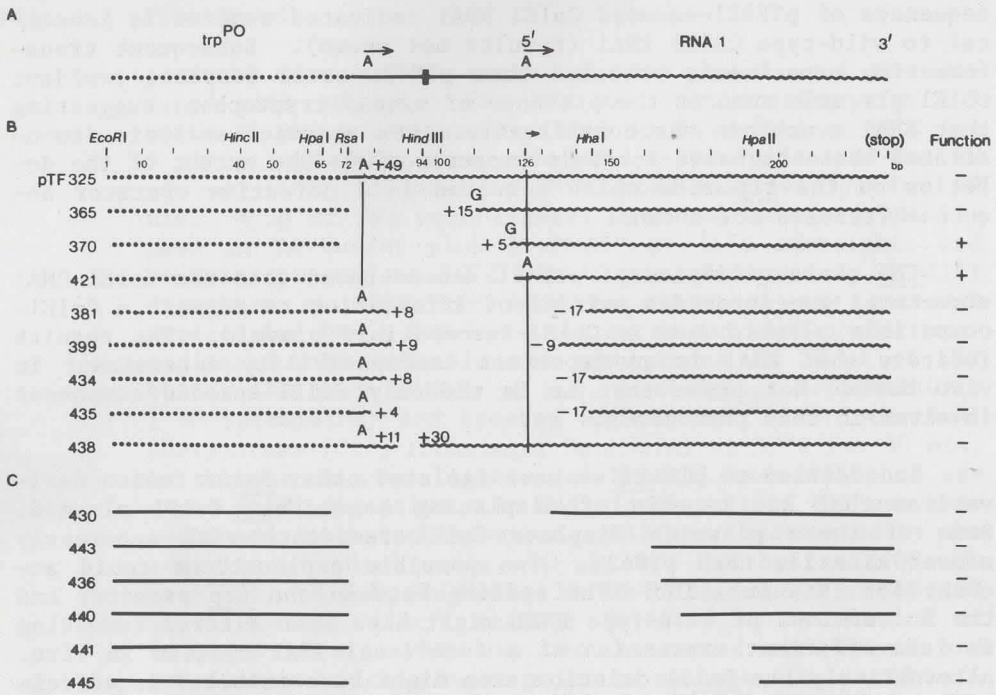


Fig. 2. Schematic diagram of the sequences of trp-RNA1 fusions. (a) trp-RNA1 fusions plasmid before Bal31 deletion. Dotted line designates trp promoter-operator sequences. The A adjacent to the promoter indicates the trp leader A. Arrows designate the direction of transcription which is capable of initiation either from the trp leader A start or the RNa1 A start depending on the extent of Bal31 digestion. The dark vertical block represents the location of the HindIII site in pTF325.

(b) Wavy lines designate transcription (left to right). For each plasmid shown, the initiating nucleotide and relative position of initiation is depicted. Plus and minus signs refer to nucleotide positions relative to the trp leader A or the correct A start of wild-type RNA1. Thus, pTF365-encoded ColE1 RNA starts with a G residue 15 nucleotides upstream from the correct A start. Several of the plasmids shown initiate RNA1 transcription from the trp leader A start. Deletion of 5' portions of RNA 1 in some plasmids has resulted in fusions of trp sequences to remaining RNA1 sequences.

Sequences of the trp-RNA1 fusion sequences were determined by analysis of transcripts produced in vitro with E. coli RNA polymerase or by M13 DNA sequencing. RNA transcripts were prepared from supercoiled DNA, purified by CsCl centrifugation, using [γ^{-32P}] GTP or ATP as labels. The labeled trp-RNA1 bands were isolated and digested with T1 ribonuclease under standard sequencing conditions (21) or subjected to limited alkaline hydrolosis. The position of each G or A residue was then determined by comparison to the known DNA sequence of the region. This approach permitted unambiguous assignment of the 5' end.

Fig. 2. (Continued)

The column labeled "Function" refers to the ability of RNA1 produced by the various plasmids to mediate ColE1 incompatibility in vivo.

(c) Deletion size estimates were made on the basis of restriction fragment analysis. Plasmid DNA was cut with EcoRV, HindII, and BamH1 in 1XTA buffer and run on a 6% (w/v) polyacrylamide gel.

fusion structures to isolate constructs that lacked biological activity as a result of inappropriate 5'-terminal sequences. Two plasmids, pTF381 and pTF399, were shown by restriction analysis to contain 58 bp and 48 bp deletions, respectively. Both plasmids have a functional trp promoter in vitro (results not shown). We have determined the RNA sequence of the RNA1 species produced by these plasmids. Each produce a hybrid RNA1 that contains sequences derived from the trp leader transcription initiation point fused to a short deletion of 5'-proximal RNA1 sequences (depicted schematically in Fig. 2b). In pTF381 the first 8 residues of the trp transcript are fused to an RNA1 body that is deleted for the first 17 nucleotides. In pTF399 9 nucleotides of the trp leader sequence are fused to an RNA1 body that is deleted for the first 9 residues. Consequently, pTF399 RNA1 is the same length as wild-type RNA1 but differs in 7 of 9 positions. The first 9 nucleotides of wild-type RNA1 are known to be in an "accessible" configuration as measured by nuclease and chemical probes of secondary structure (16). The inability of pTF399 RNA1 to mediate ColE1 incompatibility in vivo suggests that the 5'-tail region of RNA1 is critical for RNA1 function and/or stability in vivo.

In addition to these plasmids several other trp-RNA1 fusion joints have been examined at the RNA sequence level. These RNA1 structures are depicted schematically in Fig. 2b. The derivatives designated pTF434 and 435 encode hybrid RNA1 species which initiate at the trp leader A residue and contain 8 or 4 nucleotides from the trp sequence, respectively. These leaders are fused to an RNA1 body deleted for 17 nucleotides at the 5' terminus of RNA1. Like pTF381 these RNA1 species are non-functional in mediating ColE1 incompati-

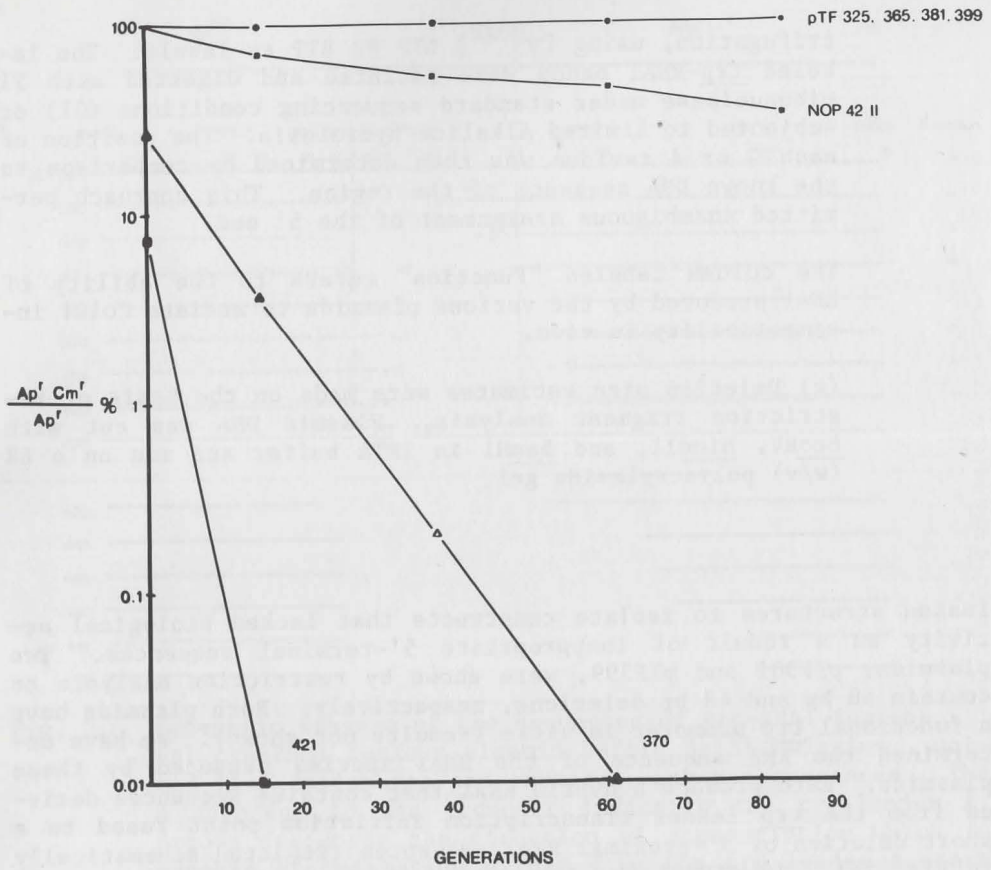


Fig. 3. Kinetics of segregation of trp-RNA1 fusion plasmids and a ColE1 resident plasmid. Plasmid DNA was transformed into the C600 strain of E. coli containing the ColE1 resident plasmid pTF345 (Cm^r), and plated on VB minimal medium E plates supplemented with 0.2% (w/v) glucose, 50 µg threonine/ml, 50 µg leucine/ml, 12 µg vitamin B1/ml, 100 µg ampicillin/ml and 30 µg chloramphenicol/ml. Zero hour points were obtained by diluting colonies in 0.85% (w/v) NaCl and plating 100 µl on VB soft agar containing 10 µg tetrazolium chloride/ml and 100 µg ampicillin/ml or 100 µg ampicillin/ml plus 30 µg chloramphenicol/ml to determine the percentage of Ap^r Cm^r/Ap^r cells. Diluted cells were also inoculated into 3 ml of VB medium containing 100 µg ampicillin/ml and allowed to grow for approximately 18 generations prior to plating. Subsequent time points were obtained by periodic dilution of cells grown into stationary phase followed by plating as above.

bility in vivo (results not shown), presumably because of alterations in secondary structure. However, effects on RNA1 stability in vivo have not been ruled out for these derivatives. Finally, pTF438 has 11 trp residues fused to a +30 RNA1 gene, i.e., an RNA1 gene is generated with an additional 41 residues. Like pTF324, pTF438 is compatible with ColE1 derivatives in vivo. A number of additional constructs with varying sized deletions in the RNA1 gene have also been isolated (Fig. 2c). None of the derivatives encodes a biologically active RNA1 species in vivo (results not shown).

One unexpected result which emerges from these experiments concerns the importance of the 5' terminal region of RNA1 for its ability to function as a replication repressor (20). Mutations affecting a limited number of residues only in the 5' "tail" region of RNA1 lead to a complete loss of activity (pTF399). More recent results using a different method of selecting RNA1 defective mutations has revealed that a single base-pair alteration in the 5' tail region can inactivate RNA1 (T. Dooley, pers. comm.). Preliminary secondary structural analysis of these mutant RNA1 species has indicated that no alterations are detectable in the 3 stems and loops. These results demonstrate that the 5'-tail of RNA1 is critical for RNA1 function. One possible role for this region is in providing a nucleation region for interaction with the primer precursor. Alternatively this region may be involved in tertiary intramolecular interactions in RNA1.

These experiments demonstrate that a small plasmid-encoded RNA molecule plays a key role in plasmid incompatibility in vivo. The basic result is consistent with the behavior of RNA1 in a purified replication system in vitro where it has been shown to negatively modulate proper processing of the replication primer (6,10). An important question concerns whether RNA1 is the sole ColE1-mediated incompatibility determinant. It should be noted that ColE1 encodes a second negative, trans-acting copy number determinant, designated the rop function (17). The rop gene encodes a small polypeptide of 63 amino acid residues which is capable of decreasing the extent of transcription initiated from the primer promoter in vivo (17,18). Recent experiments of R. Lacatena and G. Cesareni (pers. comm.) indicate that the effects of the rop gene product on primer transcription require ColE1 RNA1. Although a molecular mechanism for the action of the rop gene product is not yet at hand, several possibilities are apparent. RNA1 and the rop protein may interact directly and the resulting RNA-protein complex may be a more potent inhibition of primer processing than either alone. Alternatively, RNA1 and rop may have separate targets on the primer precursor. It is clear from deletion studies that RNA1 can function in the absence of rop (19), raising the question of whether the rop protein serves as a secondary, fine-tuning copy number control mechanism. Additional experiments using purified components in an in vitro replication system should permit their roles to be more precisely defined.

ACKNOWLEDGMENTS

We thank C. Yanofsky, D. Gelfand, and R. Somerville for strains and plasmids. This work was supported by National Institutes of Health grant GM 24212. B.P. was supported by an NIH Research Cancer Development Award.

REFERENCES

1. Donoghue, D.J., and P.A. Sharp (1978) Replication of colicin E1 plasmid DNA in vivo requires no plasmid-encoded proteins. J. Bact. 133:1287-1294.
2. Kahn, M., and D. Helinski (1978) Construction of a novel plasmid-phage hybrid: Use of the hybrid to demonstrate ColE1 DNA replication in vivo in the absence of a ColE1-specified protein. Proc. Natl. Acad. Sci., USA 75:2200-2204.
3. Conrad, S.E., and J.L. Campbell (1979) Role of plasmid-coded RNA and ribonuclease III in plasmid DNA replication. Cell 18:61-71.
4. Shepard, H.M., D.H.Gelfand, and B. Polisky (1979) Analysis of a recessive copy number mutant of the plasmid ColE1: Evidence for negative control of plasmid replication. Cell 18:267-276.
5. Itoh, T., and J. Tomizawa (1980) Formation of an RNA primer to initiation of replication of ColE1 DNA by ribonuclease H. Proc. Natl. Acad. Sci., USA 77:2450-2454.
6. Tomizawa, J., T. Itoh, G. Selzer, and T. Som (1981) Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. Proc. Natl. Acad. Sci., USA 78:1421-1425.
7. Morita, M., and A. Oka (1979) The structure of a transcriptional unit of Colicin E1 plasmid. Eur. J. Biochem. 97:435-443.
8. Pritchard, R.H., P.T. Barth, and J. Collins (1969) Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19:163-297.
9. Pritchard, R.H. (1978) Control of DNA replication in bacteria. In DNA Synthesis, I. Molineaux and M. Kohiyama, eds. Plenum Publishing Co. New York, pp. 1-26.
10. Tomizawa, J., and T. Itoh (1981) Plasmid ColE1 incompatibility determined by interaction of RNA1 with primer transcript. Proc. Natl. Acad. Sci., USA 78:6096-6100.
11. Lacatena, R.M., and G. Cesareni (1981) Base pairing of RNAI with its complementary sequence in the primer precursor inhibits ColE1 replication. Nature 294:623-626.
12. Chang, A.C.Y., and S.N. Cohen (1978) Construction and characterization of amplifiable mutlticopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bact. 134:1141-1156.
13. Roeder, W., and R.L. Somerville (1979) Cloning the trpR gene. Mol. Gen. Genet. 176:361-368.

14. O'Farrell, P.H., B. Polisky, and D.H. Gelfand (1978) Regulated expression by read through translation from a plasmid-encoded β-galactosidase. J. Bact. 134:645-654.
15. Morse, D., R.D. Mosteller, and C. Yanofsky (1969) Dynamics of synthesis, translation and degredation of trp operon messenger RNA in E. coli. Cold Spring Harbor Symp. Quant. Biol. 34:725-740.
16. Tamm, J., and B. Polisky (1983) Structural analysis of RNA molecules involved in plasmid copy number control. Nucl. Acids Res. 11:6381-6397.
17. Cesareni, G., M.A. Muesing, and B. Polisky (1982) Control of ColE1 DNA replication: The rop gene product negatively affects transcription from the replication primer promoter. Proc. Natl. Acad. Sci., USA 79:6313-6317.
18. Som, T., and J. Tomizawa (1983) Regulatory regions of ColE1 that are involved in determination of plasmid copy number. Proc. Natl. Acad. Sci., USA 80:3232-3236.
19. Twigg, A.J., and D. Sherratt (1980) Trans-complementable copy-number mutants of plasmid ColE1. Nature 283:216-218.
20. Lacatena, R.M., and G. Cesareni (1983) Interaction between RNA1 and the primer precursor in the regulation of ColE1 replication. J. Mol. Biol. 170:635-650.
21. Wrede, P., R. Wurst, J. Vournakis, and A. Rich (1979) Conformational changes of yeast tRNA phe and E. coli tRNA glu as indicated by different nuclease digestion patterns. J. Biol. Chem. 254:9608-9616.

INCOMPATIBILITY AND INCFII PLASMID REPLICATION CONTROL

Robert H. Rownd, David D. Womble, Xin-nian Dong,
Verne A. Luckow, and Ru Ping Wu*

Department of Molecular Biology
The Medical and Dental Schools
Northwestern University
Chicago, Illinois 60611

ABSTRACT

The DNA coding for replication control and incompatibility of the plasmid NR1 serves as a template in vivo and in vitro for RNA transcription in both directions. In the rightward direction, RNA synthesis begins from 2 different promoters, one of which is regulated and the other constitutive. In vivo, each of these transcripts is more than 1,000 nucleotides long, terminating near the estimated site for the origin of replication. These transcripts serve as messenger RNA for several proteins. One protein (repA1) is required for replication and another (repA2) serves as the repressor for the regulated rightward promoter. RNA synthesis in the leftward direction is constitutive and produces a single transcript of 91 nucleotides which is complementary in sequence to the rightward transcripts. This small transcript is the incompatibility product which regulates the replication of the plasmid. When the intracellular concentration of the small transcript is experimentally varied, the rate of translation of the rightward transcripts and the rate of initiation of replication (plasmid copy number) vary inversely to its concentration. The mode of action of this inhibitor RNA is likely to be formation of an RNA-RNA duplex with the rightward transcripts, thereby inhibiting the translation which would produce the required replication protein. The probability that a rightward transcript will escape interaction with the small RNA molecules and thus allow replication to initiate can be predicted from the laws of

* Present address: Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 319 Yue-Yang Road, Shanghai 20031, China.

mass action based on base-stacking free energies for the likely sequences of initial contact. The estimated intracellular RNA concentrations, based on quantitative hybridization experiments, are in agreement with those predicted from the calculated equilibrium constants for duplex formation.

INTRODUCTION

Plasmid incompatibility is the inability of 2 closely related plasmids which share the same replication control mechanism to stably coexist in the descendants of a bacterial cell. The 90-kb antibiotic resistance plasmid NR1 belongs to the FII incompatibility group and is normally maintained at about 2 copies per chromosome or 4 copies per cell (1,2). The genes required for autonomous replication of NR1 and the related IncFII plasmids R6 and R1 are located within a 2-kb region of the plasmid DNA (3-6) which retains all of the replication and incompatibility properties of the 90 -kb NR1 plasmid (3,7). The nucleotide sequences of the replication regions of NR1, R6, and R1 and of several copy number (cop) or incompatibility (inc) mutants have been determined (8-15).

The replication genes of NR1 are located within 2 contiguous PstI restriction fragments. The origin of replication is in a PstI 1.6-kb fragment (Ref. 16,17; J. Kasner and R. H. Rownd, unpub. data), while incompatibility (inc) and copy number (cop) control are in a PstI 1.1-kb fragment (3,5-7) (Fig. 1). Rightward transcription toward the replication origin is initiated in vitro at 2 promoters in the PstI 1.1-kb fragment more than 1,000 bp from the replication origin (12-14). The promoter for the 380 base RNA-C is located within the 60-bp Sau3A fragment, while the promoter for the 1,150 base RNA-A is located near the rightward end of the 332-bp Sau3A fragment (Fig. 1). RNA-C is terminated in vitro at a site about 50 bases into the 262-bp Sau3A fragment (12). Evidence presented here (and unpub. data) suggests that transcription reads through this site in vivo to form RNA-CX, which contains all the sequences in RNA-C and RNA-A (Fig. 1). The rightward transcripts could code for several proteins, called repA1, repA2, and repA3 (9), which are produced by minireplicators in minicell systems (9) or in in vitro coupled transcription/translation systems (18). The repA1 protein appears to be required for replication (3,6,9). The rightward transcripts might also serve as primers for initiation of replication at the origin (12,13). Transcription in the leftward direction produces the 91 base RNA-E (12-14,19) which is coded by the opposite strand of the 262-bp Sau3A fragment (Fig.1). It is thus complementary in sequence to the rightward transcripts. Most evidence suggests that plasmid incompatibility and replication control are mediated by this small untranslated RNA (7,11,13,19,20).

In this communication we show that the target for the RNA-E incompatibility inhibitor is within a complementary region of the

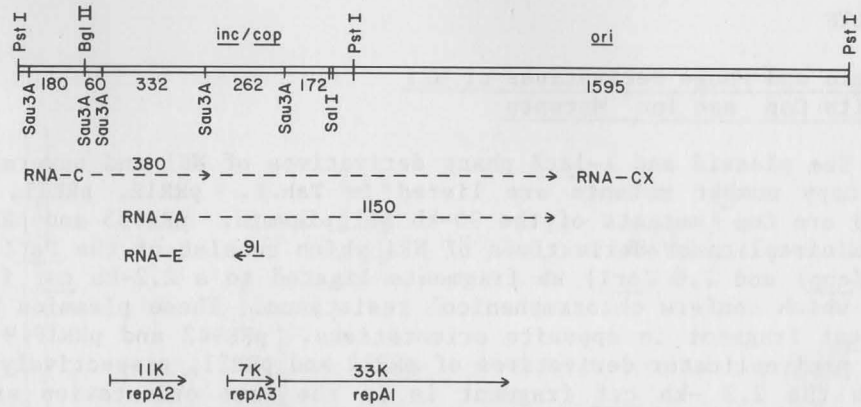


Fig. 1. Linear representation of the DNA from the replication control region of plasmid NR1. This region consists of 2 PstI restriction fragments of 1.1 kb (inc/cop) and 1.6 kb (ori). The location and direction of transcription for RNA-C, RNA-CX, RNA-A, and RNA-E are indicated by dashed arrows (12-15). The protein coding regions (9,10) are shown by the solid arrows for repA1 (33,000 d), repA2 (11,000 d), and repA3 (7,000 d). Relevant restriction sites are indicated. Minireplicators used in this study consist of these 2 PstI fragments plus a 2.2 kb PstI fragment coding for resistance to chloramphenicol (3,7). Fusion phages were constructed using the left-most SalI site in the 1.1 kb fragment, fusing RNA-CX and RNA-A to the β-galactosidase coding region of λRS205 (7). The 1.1 kb fragment and the various Sau3A fragments were also cloned into plasmid pBR322 or into the single-stranded phage M13mp10 (25).

rightward transcripts RNA-A and RNA-CX. We have examined the rates of in vivo transcription and translation from the replication region of NR1 by measuring the amount of RNA synthesis using quantitative hybridization and by assaying β-galactosidase activity after fusing the replication sequences to the lacZ gene in the lambda phage λRS205 (7). Minireplicators and λ-lacZ fusion phages from NR1 and its copy number or incompatibility mutants were also examined. The results suggest a mechanism for regulation of IncFII plasmid DNA replication. Copy number is proportional to the rate of rightward gene expression from the replication control region. This expression is regulated at the level of transcription by the repA2 protein and at the level of translation by interaction of the incompatibility inhibitor RNA-E with complementary sequences in the rightward transcripts.

RESULTS

Plasmid and Phage Derivatives of NR1 and its Cop and Inc Mutants

The plasmid and λ-lacZ phage derivatives of NR1 and several of its copy number mutants are listed in Tab.1. pRR12, pRR21, and pRR39 are Cop⁻ mutants of the 90-kb NR1 plasmid. pRR933 and pRR949 are minireplicator derivatives of NR1 which consist of the PstI 1.1 (inc/cop) and 1.6 (ori) kb fragments ligated to a 2.2-kb cat fragment which confers chloramphenicol resistance. These plasmids have the cat fragment in opposite orientations. pRR942 and pRR1919 are PstI minireplicator derivatives of pRR12 and pRR21, respectively, in which the 2.2 -kb cat fragment is in the same orientation as in pRR933. pRR5406 has a structure similar to pRR949 except that the RNA-C/CX promoter has been inactivated owing to insertion of Tn3 in the 60-bp Sau3A fragment of the parental plasmid pRR39. pRR1909 and pRR1910 were derived from pRR949 by deletion of certain Sau3A fragments. pRR1909 has deleted the RNA-C/CX promoter in the 60-bp Sau3A fragment and thus has only the RNA-A promoter for rightward transcription, whereas pRR1910 has both the RNA-C/CX and RNA-A promoters.

The PstI 1.1-kb (inc/cop) fragments from NR1, pRR12, and pRR21 have been cloned on the high copy number vector pBR322 to form pRR935 (from NR1), pRR939 (from pRR12), and pDPT261 from pRR21), respectively. pWNRR3 is a mutant of pRR935 which no longer expresses incompatibility toward NR1 and does not mediate synthesis of RNA-E owing to a "down-promoter" mutation (see below). The Sau3A 262-bp fragment of NR1 and pWNRR3, which contains only the RNA-E (leftward) promoter, has been cloned into the BamHI site of pBR322 to form pDXRR1, pDXRR2 and pDXRR4. pVLRR7 contains the Sau3A 332-bp fragment of NR1 cloned into the BamHI site of the high copy number vector pUC8. This plasmid can mediate the synthesis of the repA2 protein under the control of the lac promoter/operator. The properties of these plasmid derivatives will be discussed in further detail in the following sections.

The λlacZ phages, constructed as described previously (7), contain the part of the PstI 1.1 kb fragment to the leftward of the SalI site (Fig. 1) fused to a lacZ (β-galactosidase) coding region on the phage λRS205. In lysogens of these fusion phages β-galactosidase synthesis is under the control of the rightward NR1x replication transcripts and the prophages are named after the source of the plasmid DNA insert. For example, λ933 was derived from plasmid pRR933.

Incompatibility Properties of Plasmid Derivatives

The incompatibility properties of the minireplicators (and others not included in Tab.1) were found to be the same as those of the parent plasmids from which they were derived (Ref. 3,7; unpub.

Tab. 1. Plasmids used in this study.

PLASMID	DESCRIPTION	REFERENCE
Mutants of 90 kb NR1 plasmid		
NR1	natural isolate, IncFII resistance plasmid	21
pRR12	inc⁻ cop⁻ mutant of NR1	22
pRR21	inc⁺ cop⁻ mutant of NR1	1,6
pRR39	inc⁺ cop⁻ mutant of NR1, Tn3 inserted into the 60 bp Sau3A Fragment	C.K. Barton
Minireplicator derivatives		
pRR933	minireplicator from NR1, PstI 1.1 kb + 1.6 kb + 2.2 kb cat	3,7
pRR949	minireplicator from NR1, PstI 1.1 kb + 1.6 kb + 2.2 kb cat (reverse orientation)	7
pRR942	minireplicator from pRR12, PstI 1.1 kb + 1.6 kb + 2.2 kb cat	3,7
pRR1919	minireplicator from pRR21, PstI 1.1 kb + 1.6 kb + 2.2 kb cat	7
pRR5406	minireplicator from pRR39, similar structure to pRR949	C.K. Barton
pRR1909	Sau3A deletion of pRR949, retains the 332 bp and 262 bp fragments	A.M. Easton
pRR1910	Sau3A deletion of pRR949, retains the 60 bp, 332 bp, and 262 bp fragments	A.M. Easton
PstI fragments cloned on high copy number vectors		
pBR322	cloning vector	23
pRR935	pBR322 plus the PstI 1.1 kb inc fragment of NR1	7
pRR939	pBR322 plus the PstI 1.1 kb inc fragment of pRR12	7
pDPT261	pBR322 plus the PstI 1.1 kb inc fragment of pRR21	6,7
pWNRR3	inc⁻ mutant of pRR935	R.P. Wu
Sau3A fragments cloned on high copy number vectors		
pDXRR1	pBR322 plus the Sau3A 262 bp fragment of pWNRR3, + orientation	X. Dong
pDXRR2	pBR322 plus the Sau3A 262 bp fragment of pWNRR3, - orientation	X. Dong
pDXRR4	pBR322 plus the Sau3A 262 bp fragment of NR1, - orientation	X. Dong
pUC8	cloning vector	24
pVLRR7	pUC8 plus the Sau3A 332 bp fragment of NR1, + orientation	V.A. Luckow
λ-lacZ phage derivatives		
λ933	λRS205 derivative containing RNA-C and RNA-A promoters of pRR933 fused to lacZ	7
λ942	λRS205 derivative containing RNA-C and RNA-A promoters of pRR942 fused to lacZ	7
λ1919	λRS205 derivative containing RNA-C and RNA-A promoters of pRR1919 fused to lacZ	7
λ1909	λRS205 derivative containing RNA-A promoter of pRR1909 fused to lacZ	X. Dong
λ1910	λRS205 derivative containing RNA-C and RNA-A promoters of pRR1910 fused to lacZ	X. Dong

data). All of the NR1, pRR21, and pRR39 derivatives were incompatible with other derivatives of these plasmids in all possible combinations. Various pRR12 (*inc*$^-$ *cop*$^-$)$_+$ minireplicators were compatible with NR1 (*inc*$^+$ *cop*$^+$) and pRR21 (*inc*$^+$ *cop*$^-$) derivatives and had only weak incompatibility against other pRR12 minireplicators. However, when cloned on a high copy-number vector, the PstI 1.1-kb or Sau3A 262-bp fragments from pRR12 displayed strong incompatibility against pRR12 minireplicators, but were compatible with NR1 Inc$^+$ derivatives. The corresponding derivatives of NR1 or pRR21 displayed strong incompatibility against NR1, pRR21, or pRR39 derivatives, but were compatible with pRR12 derivatives even though the cloned *inc* fragments were present on high copy-number vectors. Thus, pRR12 is mutant simultaneously in both the incompatibility inhibitor and its target (3,7).

Measurement of In Vivo Transcription Rates

In order to examine the rates of expression of the NR1 replication control genes (Fig. 1) quantitative hybridization of (^{3}H)-labeled RNA was used to measure transcription rates in either the rightward or leftward direction independently. The DNA "probes" were composed of the single-stranded phage M13mp10 (25) containing specific Sau3A fragments from the replication region of NR1. The 332-bp fragment in the "+" orientation will hybridize with RNA-C (see Fig. 1), the 262-bp fragment in the "+" orientation will hybridize with RNA-CX and RNA-A, the 262-bp fragment in the "-" orientation will hybridize with RNA-E, and the 332-bp fragment in the "-" orientation measures only nonspecific binding (unpub. data). In vivo transcription rates were measured in cells harboring derivatives of NR1 or its copy mutants pRR12 (*inc*$^-$ *cop*$^-$) and pRR21 (*inc*$^+$ *cop*$^-$) (Tab.2). λ933, λ942, and λ1919 are λ-*lac* derivatives of the minireplicators pRR933, pRR942, and pRR1919, respectively, while pRR935, pRR939, and pDPT261 are pBR322 derivatives into which the corresponding PstI 1.1-kb fragments have been cloned. This series allows measurement of in vivo transcription when the templates are all maintained at the same low copy number (λ-*lacZ* lysogens), when they are replicating at their natural copy numbers (minireplicators) or when they are maintained at the same high copy number (pBR322 derivatives).

The corresponding λ-*lacZ* (λ933 and λ942), minireplicator (pRR933 and pRR942), and pBR322 (pRR935 and pRR939) derivatives of NR1 and the *inc*$^-$ *cop*$^-$ pRR12 are transcribed at equivalent levels per gene copy. For example, the relative transcription rate per copy of λ933 and λ942 for RNA-E was 2.20 and 2.03, for RNA-C was 0.22 and 0.24, and for RNA-CX plus RNA-A was 1.00 and 1.10, respectively (Tab.2). Except for the λ-*lacZ* lysogens, the *inc*$^+$ *cop*$^-$ pRR21 derivatives have a 10-fold increase in the level of both RNA-C and RNA-CX plus RNA-A sequences whereas they have approximately the same relative transcription rate per copy for RNA-E as the NR1 and pRR12 derivatives. RNA-C transcription is also elevated 10-fold in the

Tab. 2. In vivo transcription rates.[a]

Template	*inc*	*cop*	Copies per Chromosome[c]	Relative Transcription Rate per Copy[b] RNA-E	RNA-C	RNA-CX + RNA-A	(RNA-CX + RNA-A)/RNA-C
λ933	+	+	1.0	2.20	0.22	1.00	4.6
pRR933	+	+	2.2	2.77	0.25	0.19	0.76
pRR935	+	+	40	4.94	0.24	0.14	0.60
λ942	-	-	1.0	2.03	0.24	1.10	4.6
pRR942	-	-	7.3	3.21	0.26	0.17	0.64
pRR939	-	-	40	3.41	0.24	0.14	0.59
λ1919	+	-	1.0	6.02	2.50	2.20	0.88
pRR1919	+	-	9.9	1.14	2.44	1.68	0.69
pDPT261	+	-	40	1.52	2.52	1.63	0.65
pWNRR3	-	d	40	0.08	0.27	0.30	1.11

[a] Exponential phase cells were pulse-labeled for 2.0 min with 5-[^{3}H]-uridine at 20 μCi/ml, 25 Ci/mmole, followed by quick killing with a frozen mixture containing chloramphenicol and sodium azide (26). RNA was extracted as described (26) yielding about 250 μg at greater than 10^5 cpm/μg. RNA (about 5 x 10^6 cpm) was quantitatively hybridized to a vast excess of single-stranded DNA fixed to nitrocellulose using published methods (26, 27). The DNA was from the single-stranded phage M13mp10 (25) carrying the 332bp or 262bp Sau3A fragments from the NR1 replication segment (Fig. 1) inserted in either orientation relative to the viral strand (unpublished data). The filters were treated with RNase prior to measuring the bound cpm. Non-specific binding (about 100 cpm) was assayed for each RNA sample using single-stranded M13mp10 DNA alone. The method is quantitative, directly proportional to RNA input, and specific (unpublished data).

[b] The fraction of ^{3}H-labeled RNA which bound to specific DNA probes was normalized to the number of uracil residues in each transcript in the portion of the transcript complementary to the probe and divided by the copy number of the template. This gives the relative transcription rate for each type of RNA, with the value for (RNA-CX + RNA-A) in λ933 called 1.00. The actual fraction bound for (RNA-CX + RNA-A) in λ933 was $1.56 \times 10^{-4} \pm 0.30 \times 10^{-4}$.

[c] The relative copy numbers of plasmids were estimated from the covalently closed circular plasmid DNA to chromosome DNA in CsCl - ethidium bromide gradients (1) or from the specific activity of chloramphenicol acetyltransferase in cell extracts (7, 28).

[d] Cannot form a mir replicator.

pRR21 λ-*lacZ* lysogen (λ1919). The smaller relative increase in RNA-CX plus RNA-A sequences of λ1919 is explained below. DNA sequence analysis has shown that pRR21 has a mutation in the RNA-C promoter (unpublished data), which explains the 10-fold increase in the rate of transcription of RNA-C and RNA-CX. In most cases, RNA-E is produced in excess compared to RNA-CX plus RNA-A (Tab.2). However, compared to its parent pRR935 (pBR322 plus the 1.1-kb PstI fragment of NR1), RNA-E is reduced more than 50-fold from the mutant plasmid pWNRR3, which does not express incompatibility (exclusion) toward either wild-type plasmids or other incompatibility mutants (unpub. data). DNA sequence analysis has revealed a "down" mutation in the RNA-E promoter of pWNRR3. Thus, RNA-E is the incompatibility inhibitor of IncFII plasmids.

The RNA-A Promoter is Repressed by the RepA2 Protein

The levels of transcription and translation of the rightward transcripts can be monitored using the λ-lacZ lysogens. λ-lacZ fusion phages were constructed from the Sau3A deletion derivatives pRR1909 and pRR1910 (Tab.1). The λ1909 derivative lacks the Sau3A 60-bp fragment containing the RNA-C promoter and thus does not produce RNA-C, RNA-CX, or repA2 protein. RNA-A is the only rightward NR1 transcript from λ1909. λ1910 has the Sau3A 60-bp fragment and therefore makes RNA-C, RNA-CX, and repA2. Lysogens with λ1909 or λ1910 were compared to the λ1919 lysogen, which has an increased level of RNA-C and RNA-CX transcription. When repA2 protein is provided in trans by plasmid pVLRR7, which has the repA2 gene cloned into the vector pUC8 (Tab.1), β-galactosidase activity from the RNA-A promoter of λ1909 is completely repressed (Tab.3). In the absence of extra repA2 protein β-galactosidase activity from all lysogens is proportional to the sum of RNA-CX plus RNA-A, while in the presence of excess repA2 protein it is proportional to RNA-CX alone. Therefore, when RNA-A is repressed, RNA-CX provides a basal level of rightward transcription.

When the repA2 gene dosage is high enough to repress completely RNA-A transcription, the ratio of (RNA-CX + RNA-A)/RNA-C should reflect the frequency of extension of RNA-C to form RNA-CX. This ratio is 0.6-0.7 for all the minireplicators and pBR322 derivatives (Tab.2), suggesting that sufficient repA2 protein is present to repress RNA-A transcription for all these plasmids, including the minireplicator derivative pRR933 from the wild-type, low copy-number plasmid NR1. The 6- to 7-fold increase in this ratio for the λ933 and λ942 lysogens is due to the derepression of RNA-A synthesis at the low copy number of one per chromosome. This increase is much smaller in the λ1919 lysogen because most of the rightward transcription comes from synthesis of RNA-C and RNA-CX owing to the mutation in the RNA-C promoter. At the low copy number in the lysogens, the endogenous amount of repA2 protein produced by λ933 and

Tab. 3. Repression of the RNA-A promoter by trans repA2 protein.

	Relative Transcription Rate[a]		β-galactosidase Activity[b]	
Lysogen	RNA-CX + RNA-A	RNA-C	-repA2	+repA2
λ1909	1.02	0.00	377	0
λ1910	0.66	0.36	163	58
λ1919	2.20	2.50	965	1102

[a] Determined in the absence of additional repA2 protein as described in Table 2.

[b] Miller Units (ref. no. 29). The additional repA2 protein was supplied in trans by coresident plasmid pVLRR7.

λ942 is apparently insufficient to repress completely the RNA-A promoter. However, repression of RNA-A synthesis is established when the repA2 gene dosage is doubled, as in the case of the minireplicator pRR933. Thus, the transcription data in Tab. 2 suggests that almost complete repression of RNA-A synthesis is achieved at the normal copy number of the minireplicators. The fully derepressed level of RNA-A transcription could be estimated from the λ1909 lysogen (Tab.3) and from the minireplicator pRR5406 (data not shown) which do not make any repA2 protein. In the wild-type plasmid derivatives, the strengths of the promoters can be ranked as RNA-E > derepressed RNA-A > RNA-C > repressed RNA-A.

RNA-E Inhibits and RNA-A Stimulates repA1 Gene Expression and Plasmid Copy Number

In order to examine the effects of RNA-A, RNA-E, and repA2 protein on gene expression and copy number in trans, plasmids which synthesize these products were introduced into either a λ933 lysogen or a strain harboring the pRR933 minireplicator. The derivations of the coresident plasmids are presented in Tab.1, and the trans products they produce were assayed independently (Tab.2 and unpub. data). All are derivatives of pBR322 carrying the PstI 1.1-kb or Sau3A 262-bp or 332-bp fragments from the replication region of NR1 or its incompatibility mutants. The RNA-E promoter, which is nonfunctional in pWNRR3 derivatives, is the only promoter on the Sau3A 262-bp fragment (12,13). Therefore, when the 262-bp fragment of pWNRR3 is cloned into the BamHI site of pBR322, "RNA-A" or "RNA-E" hybrid RNA sequences are synthesized by transcription from the tetracycline resistance gene (tet) promoter (Fig. 2). The results in Tab.4 show that excess RNA-E both inhibited β-galactosidase synthesis from λ933 and reduced the copy number of a coresident pRR933 plasmid. RNA-E produced from an incompatibility mutant (pRR939) or as a hybrid from the tetracycline resistance promoter (pDXRR2) was less effective than wild-type RNA-E (pRR935, pDXRR4). Excess RNA-A both stimulated β-galactosidase activity in the lysogen and raised the copy number of a coresident minireplicator. Presumably this resulted from titration of the RNA-E synthesized from the lysogenic phage or the minireplicator by the trans RNA-A. Thus, the rightward transcript sequences from the 262-bp Sau3A fragment (RNA-A and RNA-CX sequences) must be the target for the incompatibility repressor RNA-E. The plasmid pDXRR5 (Fig. 2) had little or no transcription from either strand of the 262-bp Sau3A fragment owing to deletion of the tet gene promoter after cleavage of pDXRR1 with HindIII, S1 digestion, and ligation. pDXRR5 had little effect on either β-galactosidase synthesis or pRR933 copy number. Thus, the presence of the DNA sequences alone had no effect in trans, showing that it is the rightward RNA transcripts and not the DNA which is the target of RNA-E inhibition. An excess of repA2 protein from plasmid pVLRR7 reduced β-galactosidase activity in the lysogen (Tab.2). However, in the presence of excess repA2 protein, excess RNA-E or RNA-A had their expected inhibitory or stimulatory effects, respectively.

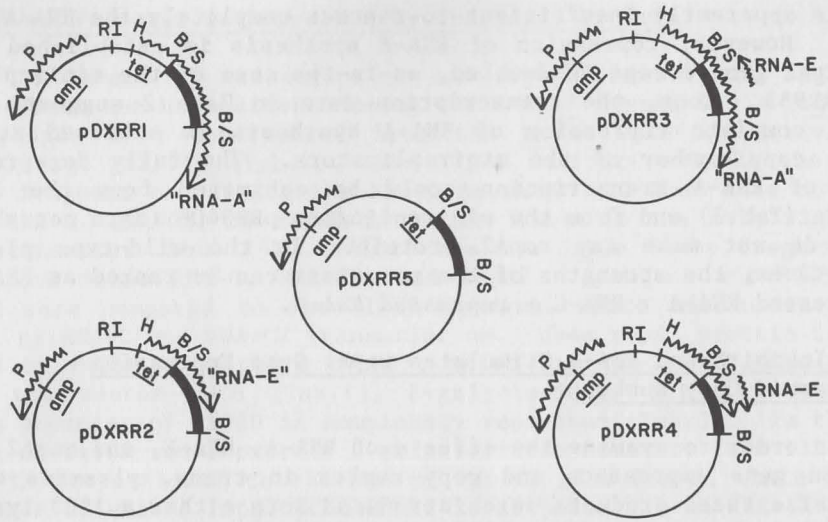


Fig. 2. Maps of pBR322 clones carrying the 262 bp Sau3A fragments from the replication control region of pWNRR3 (pDXRR1, pDXRR2, and pDXRR5) or NR1 (pDXRR3 and pDXRR4). Insertions were in either orientation at the BamHI site in the tetracycline resistance gene. Transcription products are indicated as wavy arrows. Restriction sites are: P, PstI; RI, EcoRI; H, HindIII; B/S, BamHI-Sau3A fusion.

Intracellular RNA Concentrations

The stabilities of RNA-C, RNA-A, and RNA-E were also examined by hybridization after a pulse-chase experiment. Each was unstable with a half-life of less than 10% of a generation (data not shown). If it is assumed that these transcripts behave similarly to normal mRNA (36), their intracellular concentrations can be estimated from the hybridization data (Fig. 3). The levels of RNA-C and RNA-E were constant per plasmid copy over the entire range of copy numbers. The level of derepressed RNA-A per plasmid copy was also constant in the case of plasmids which have deleted or inactivated the RNA-C promoter and do not synthesize the repA2 repressor protein. The level of RNA-CX plus RNA-A per copy was also constant above about 5 copies per cell. At lower copy numbers the level of RNA-CX plus RNA-A increased owing to a lower level of synthesis of repA2 and approached that expected for the constant level of RNA-CX plus the fully derepressed level of RNA-A. The normal copy number of NR1 is about 4.4 copies per cell. The dotted lines extrapolated to the ordinate represent the unregulated, or fully derepressed, levels of each transcript.

Tab. 4. Regulation of gene expression and copy-number.

Coresident	trans Products[a]	Relative β-galactosidase Activity λ933	Relative β-galactosidase Activity pKK933	Relative Copy Number pKK933
none	none	0.83		1.0
pBR322	none	1.00		1.0
pDXRR1	A_{tet}	3.00		2.5
pDXRR2	E_{tet}	0.30		0.7
pDXRR4	E	0.30		0.0[b]
pDXRR5	none	1.21		1.1
pVLRR7	repA2	0.29		0.8
pWNRR3	repA2, A	0.98		3.2
pRR935	repA2, A, E	0.02		0.0[b]
pRR939	repA2, A_{12}, E_{12}	.0.19		0.7

[a] A=sequences of RNA-A, A_{tet}="tet-A hybrid RNA, E=RNA-E, E_{tet}="tet-E hybrid RNA, A_{12} and E_{12} are the transcripts from the *inc*$^{+}$ pRR12 derivative, and repA2 is the protein coded by RNA-C (Fig. 1).

[b] The coresident plasmid expressed incompatibility against pRR933, effectively reducing its copy number to 0.

DISCUSSION

Derivatives of the inc⁻ cop⁻ mutant pRR12 are transcriptionally indistinguishable from the wild-type NR1, but have stimulated gene expression (lysogen) (Ref.7; unpub. data) and increased copy number (minireplicator) (3,7). This suggests that the pRR12 incompatibility mutation affects a post-transcriptional step in replication control. There is a single base substitution in pRR12 (Ref.9; unpub. data) which results in a complementary change in sequence to both RNA-E and the rightward transcripts RNA-CX and RNA-A.

Derivatives of the inc⁺ cop⁻ pRR21 have a 10-fold increase in rightward transcription and the λ1919 lysogen also mediates increased β-galactosidase synthesis. The copy-number increase for this mutant is likely a result of the increased rate of rightward transcription from the RNA-C promoter, which increases the level of RNA-C and RNA-CX.

Increased levels of trans RNA-E inhibit rightward gene expression and lower the copy number of a coresident minireplicator. This inhibition shows incompatibility group specificity as seen by the difference between the wild-type NR1-derivative pRR935 and the mutant pRR12-derivative pRR939 (Tab.4). Increased levels of trans RNA-A stimulate both rightward gene expression and increase the copy number of a coresident minireplicator, probably by binding the endogenous RNA-E synthesized by the lysogen or the minireplicator. Other experiments have shown that the transcription rates themselves are not affected by excess trans RNA-A or RNA-E (unpub. data). Together with the results for the pRR12 derivatives, which show no alteration in transcription rates, these observations are indicative of post-transcriptional regulation. It is likely that RNA-E inhibits translation of the rightward transcripts by forming a complementary RNA-RNA duplex. The repA2 protein also reduces rightward gene expression by repression of transcription from the RNA-A promoter. These interactions suggest that IncFII plasmids have 2 basic mechanisms of replication control.

One mechanism of control is through derepression of RNA-A synthesis. This will occur if the plasmid copy number falls below the wild-type level and the repA2 concentration is decreased owing to gene dosage effects. The active form of the repA2 repressor appears to be a tetramer (unpub. data). Thus, a decrease in repA2 concentration would result in a considerably larger decrease in its repressor activity. The derepressed promoter for RNA-A is stronger than the RNA-CX promoter and the increased level of total rightward transcription under these conditions would increase the probability of replication. The copy mutant pRR21 has a higher level of transcription illustrating how additional rightward transcription can increase the rate of replication. Other experiments have shown that plasmids with derepressed RNA-A transcription, such as pRR39, or increased transcription owing to fusion to some other strong promoter also have high copy numbers (unpub. data).

A second mechanism of control is mediated by the interaction of the incompatibility inhibitor with the rightward transcripts to inhibit translation of repA1. This would occur at all plasmid copy numbers but would be most effective at normal copy numbers or above when the RNA-E and rightward transcript concentrations are higher. In this situation the RNA-A promoter is repressed and a basal level of rightward transcription is provided by RNA-CX (Fig.3). Additional repA2 protein has little effect on plasmid copy number (Tab.4).

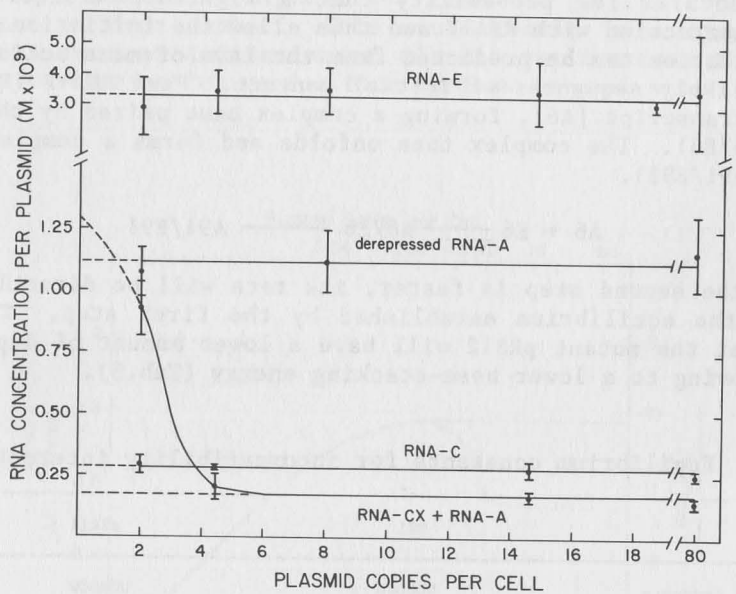


Fig. 3. Intracellular RNA concentration as a function of plasmid copy number. The concentrations per plasmid copy were estimated from the hybridization data used to produce Tab. 2 and Tab. 3 as well as data from additional experiments (not shown). Half of pulse-labeled RNA is unstable messenger RNA, while the average E. coli cell contains 2 chromosome equivalents and has about 1.73×10^6 molecules (as rNMP) of mRNA (36) in a volume of 1.6×10^{-15} liters (31). The intracellular concentration of any unstable RNA molecule can be estimated by: $\frac{(F) \times 2 \times 1.73 \times 10^6}{(H) \times 6.023 \times 10^{23} \times 1.6 \times 10^{-15}}$ = mole/liter where F is the fraction of cpm bound to a probe with H bp of homology to the transcript. The solid lines connect the points for the measured values (mean ± standard deviation) while the dashed lines extrapolate the data to the Y-axis to represent the unregulated level of synthesis. For reference, one molecule per cell gives a concentration of 1.04×10^{-9} M.

Replication control is provided by the interaction of RNA-E with the rightward transcripts which would be primarily RNA-CX. Copy number mutations which affect incompatibility simultaneously change the sequence of RNA-E and the rightward transcripts. The base changes in these mutants are located in or next to possible 6-base loops of proposed secondary structures for both RNA-E and the rightward transcripts (9-11,13). A comparison of the loop sequences of NR1 and pRR12 is shown in Tab.5 If it is assumed that the initial interaction between RNA-E and the rightward transcripts occurs by base pairing of the 6-base loops (as suggested by the mutations), then base-stacking free energies (30) and equilibrium constants for this pairing, corrected to in vivo ionic conditions (31), can be calculated (Tab.5). The probability that a rightward transcript will escape interaction with RNA-E and thus allow the initiation of plasmid replication can be predicted from the laws of mass action assuming the likely sequences of initial contact. Free RNA-E (E6) binds to free transcript [A6], forming a complex base paired by the 6-base loops (A6/E6). The complex then unfolds and forms a complete 91 bp duplex (A91/E91).

$$A6 + E6 \rightleftharpoons A6/E6 \longrightarrow A91/E91$$

If the second step is faster, its rate will be directly dependent on the equilibrium established by the first step. This suggests that the mutant pRR12 will have a lower amount of duplex formation, owing to a lower base-stacking energy (Tab.5).

Tab. 5. Equilibrium constants for incompatibility interactions.[a]

	NR1	pRR12
Loop Sequence	UUGGCG	UUUGCG
ΔG° (kcal/mole)	-13.6	-9.80
Kd (M)	1.10×10^{-10}	6.67×10^{-8}
[RNA-E] (M)	1.51×10^{-8}	5.01×10^{-8}
[RNA-CX + RNA-A] (M)	9.10×10^{-10}	2.65×10^{-9}

[a] The RNA sequence shown is the 6-base loop in the proposed secondary structure for the rightward transcripts (Fig. 5A) (9-11, 13). The proposed structure for RNA-E has a complementary loop sequence which can pair with the loop of the rightward transcripts (Fig. 5B). Base-stacking free energies (ΔG°) for this pairing (30) corrected to in vivo ionic conditions (0.23 M NaCl, + 0.5 kcal/mole per base-pair; ref. no. 30, 31) are presented and standard dissociation constants (Kd) are calculated:

$$Ka = \frac{[A6/E6]}{[A6]\,[E6]} = 1/Kd = e^{-\Delta G^\circ/RT}$$

See text for definition of A6, E6, and A6/E6. The RNA concentrations per cell are taken from Fig. 3 for pRR933 (NR1 derivative, 4.4 copies/cell) and pRR942 (pRR12 derivative, 14.6 copies/cell).

From the data in Fig. 3 the intracellular RNA concentrations expected for the wild-type plasmid NR1 can be calculated as a function of plasmid copies per cell. The fraction of rightward RNA not bound by RNA-E can then be calculated using the NR1 equilibrium constant in Tab. 5 (Fig. 4, dashed line). The total free rightward RNA concentration [A6], can then be calculated as a function of copy number (Fig. 4, solid line). The repression of RNA-A transcription above 4 copies per cell would result in the lower level of [A6] indicated by the alternate dashed-dotted line. The estimated free rightward RNA concentration can be considered to reflect the probability that a rightward transcript will escape regulation by RNA-E, resulting in translation and the initiation of replication. At normal NR1 copy numbers or higher, the level of [A6] is constant and independent of copy number. This would result in a copy number-independent rate of initiation of plasmid replication, thus automatically correcting for random fluctuations in pool size due to un-

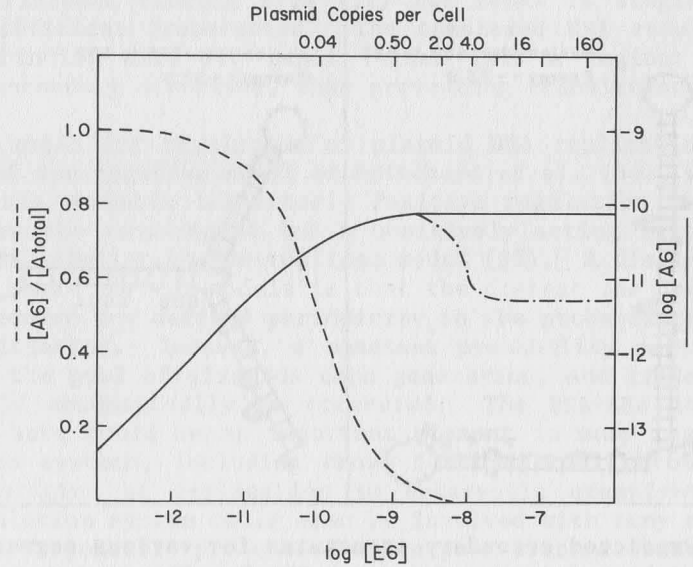


Fig. 4. Calculated interaction of RNA-E with the rightward transcripts RNA-CX and RNA-A as a function of plasmid NR1 copy number per cell. Using the RNA concentration estimates in Fig. 3 and the dissociation constant for NR1 in Tab. 5, the unbound fraction of rightward RNA, [A6]/[A total] (dashed line), and the molar values for the free rightward RNA concentration, [A6], were calculated as a function of plasmid copy number. The solid line was calculated assuming a constant ratio of rightward and leftward transcription. The alternate dashed-dotted line takes into account the repression of RNA-A transcription as the plasmid copy number increases above the NR1 copy number of 4 per cell.

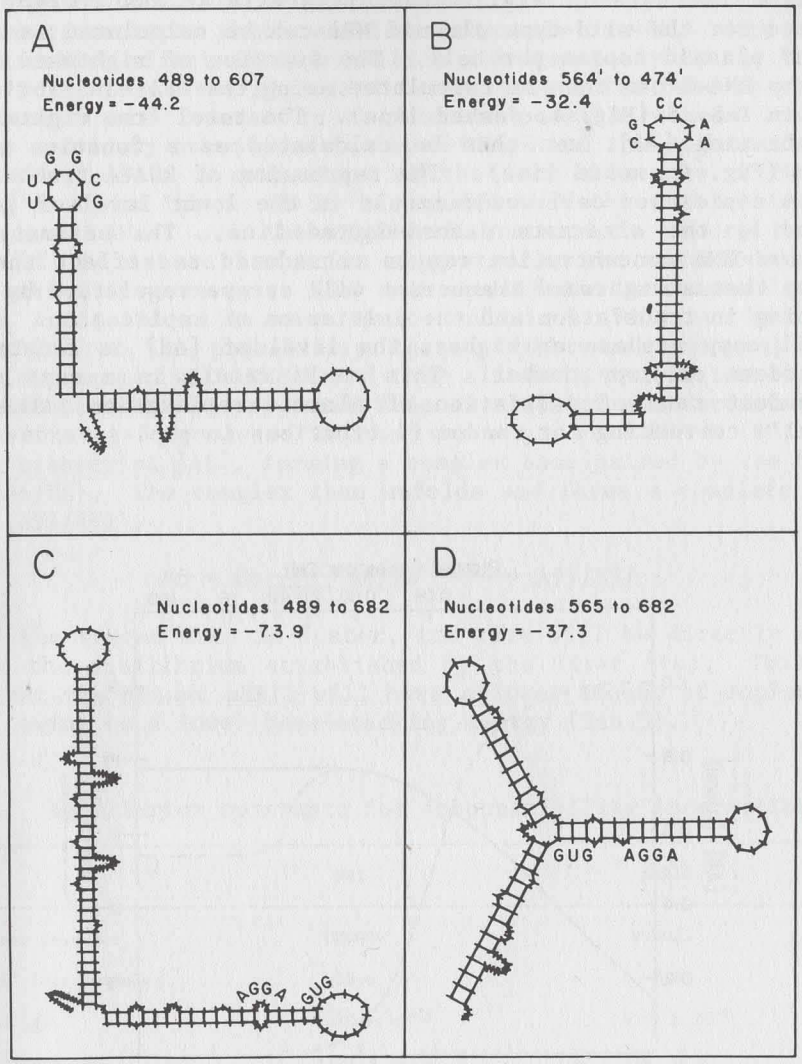


Fig. 5. Predicted secondary structures for various segments of the rightward transcripts and RNA-E. The target segments of rightward RNA and RNA-E which are complementary are shown in A and B, respectively. The 6-base loops are the presumed sites of initial interaction. The translation start site for the repA1 coding sequence is shown in the absence (C) or presence (D) of RNA-E. The base numbering is that of Rosen et al. (9), which starts at the leftward end of the 60 bp Sau3A fragment in Fig. 1 and increases to the rightward. The structures were predicted with the computer program of Zuker and Steigler (37), which uses the base-stacking and destabilizing rules of Salser (38).

equal plasmid partitioning at cell division or premature initiation of replication. Similar calculations for the copy mutants pRR12, pRR21, and pRR39 predict higher levels of [A6] than for NR1 which would result in a higher steady-state plasmid copy number (not shown). The higher level of [A6] for pRR12 is the result of a weaker RNA-RNA interaction. The higher levels for pRR21 and pRR39 are the result of a higher level of rightward transcription.

How might the formation of a duplex between RNA-E and the rightward transcripts inhibit translation and replication? If the repA1 protein is a required initiator protein, then preventing translation of its mRNA would inhibit replication. Possible secondary structures for the rightward transcripts and RNA-E were examined by computer analysis (Fig. 5). If a full 91-base duplex is formed between RNA-E and the rightward transcript, then 91 bases of the rightward RNA would be prevented from further secondary structure interactions with other regions of its own sequence. The predicted secondary structures for rightward RNA in the absence or presence of RNA-E are different. The free rightward transcripts could fold such that the ribosome binding site (32) for repA1 is single-stranded, allowing efficient translation. The complexed RNA results in the ribosome binding site for repA1 folded into a region of double-stranded secondary structure, thus preventing translation (33).

This model for regulation of plasmid DNA replication includes elements of the negative model of Pritchard et al. (34), where RNA-E would be the unstable inhibitor. Positive regulation is also suggested from the requirement for a positively acting initiator protein, repA1, similar to the replicon model (35). A distinct difference from these earlier models is that the present one does not predict or require any defined periodicity in the probability of replication initiation. Instead, a constant probability per cell would duplicate the pool of plasmids each generation, and random fluctuations would automatically be corrected. The RNA-RNA interactions described here could be an important element in many regulated DNA replication systems, including those for bacterial chromosomes and multiple origins of replication in eukaryotic organisms. Such a novel regulation system could also be involved with many other kinds of cellular regulatory processes, where post-transcriptional regulation might be desirable.

ACKNOWLEDGMENTS

We would like to thank Malcolm E. Winkler for advice on RNA extraction and hybridization; Steven C. Kowalczykowski for helpful discussions of RNA-RNA base-stacking interactions, Alan M. Easton, Padmini Sampathkumar, and Carolyn R. Barton for permission to cite their unpublished data; and Joseph E. Gerwin, for his excellent technical assistance. This work was supported in part by U.S. Public Health Services research grants GM14398 and GM30731, and by

grants from the Otho S.A. Sprague Memorial Institute and the Arlo M. Bane fund. The secondary structure analysis was performed with funds provided by the Vogelback Computing Center, Northwestern University.

REFERENCES

1. Womble, D.D., D.P. Taylor, and R.H. Rownd (1977) A method for obtaining more-accurate covalently closed circular plasmid-to-chromosome ratios from bacterial lysates by dye-buoyant density centrifugation. J. Bact. 130:148-153.
2. Rownd, R.H., and D.D. Womble (1978) Molecular nature and replication of R factors. In R factor, Drug Resistance Plasmid, S. Misuhashi ed. University of Tokyo Press, Tokyo, pp. 161-193.
3. Miki, T., A.M. Easton, and R.H. Rownd (1980) Cloning of replication, incompatibility, and stability functions of R plasmid NR1. J. Bact. 141: 87-99.
4. Molin, S., and K. Nordstrom (1980) Control of plasmid R1 replication: Functions involved in replication, copy number control, incompatibility, and switch-off of replication. J. Bact. 141:111-120.
5. Timmis, K.N., I. Andres, and P.M. Slocombe (1978) Plasmid incompatibility: cloning analysis of an incFII determinant of R6-5. Nature (London) 273: 27-32.
6. Taylor, D.P., and S.N. Cohen (1979) Structural and functional analysis of cloned DNA segments containing the replication and incompatibility regions of a miniplasmid derived from a copy number mutant of NR1. J. Bact. 137:92-104.
7. Easton, A.M., and R.H. Rownd (1982) The incompatibility product of IncFII R Plasmid NR1 controls gene expression in the plasmid replication region. J. Bact. 152:829-839.
8. Rosen, J., H. Ohtsubo, and E. Ohtsubo (1978) The nucleotide sequence of the region surrounding the replication origin of an R100 resistance factor derivative. Mol. Gen. Genet. 171:287-293.
9. Rosen, J., T. Ryder, H. Inokuchi, H. Ohtsubo, and E. Ohtsubo (1980) Genes and sites involved in replication and incompatibility of an R100 plasmid derivative based on nucleotide sequence analysis. Mol. Gen. Genet. 179:527-537.
10. Stougaard, P., S. Molin, K. Nordstrom, and G. Hansen (1981) The nucleotide sequence of the replication control of the resistance plasmid R1drd-19 Mol. Gen. Genet. 181:116-122.
11. Brady, G., J. Frey, H. Danbara, and K.N. Timmis (1983) Replication control mutations of plasmid R6-5 and their effects on interactions of the RNA-I control element with its target. J. Bact. 154:429-436.
12. Easton, A.M., P. Sampathkumar, and R.H. Rownd (1981) Incompatibility of IncFII R plasmid NR1. In The Initiation of DNA Replication, D.S. Ray, ed. Academic Press, Inc., New York, pp. 125-141.

13. Rosen, J., T. Ryder, H. Ohtsubo, and E. Ohtsubo (1981) Role of RNA transcripts in replication incompatibility and copy number control in antibiotic resistance plasmid derivatives. Nature (London) 290:794-797.
14. Lurz, R., H. Danbara, B. Ruckert, and K.N. Timmis (1981) Plasmid replication functions. VII. Electron microscopic localization of RNA polymerase binding sites in the replication control region of plasmid R6-5. Mol. Gen. Genet. 183:490-496.
15. Chan, P.T., and J. Lebowitz (1982) Mapping of RNA plymerase binding sites in R12 derived plasmids carrying the replication-incompatibility region and the insertion element IS1. Nucleic Acids Res. 10:7295-7311.
16. Ohtsubo, E., J. Feingold, H. Ohtsubo, S. Mickel, and W. Bauer (1977) Unidirectional replication in Escherichia coli of three small plasmids derived from R factor R12. Plasmid 1:8-18.
17. Synenki, R.M., A. Nordheim, and K.N. Timmis (1979) Plasmid replication functions. III. Origin and direction of replication of a "mini" plasmid derivative from R6-5. Mol. Gen. Genet. 168:27-36.
18. Brawner, M.E., and S.R. Jaskunas (1982) Identification of polypeptides encoded by the replication region of resistance factor R100. J. Mol. Biol. 159:35-55.
19. Stougaard, P., S. Molin, and K. Nordstrom (1981) RNAs involved in copy-number control and incompatibility of plasmid R1. Proc. Natl. Acad Sci., USA 78:6008-6012.
20. Light, J., and S. Molin (1983) Post-transcriptional control of expression of the repA gene of plasmid R1 mediated by a small RNA molecule. EMBO J. 2:93-98.
21. Rownd, R., R. Nakaya, and A. Namamura (1966) Molecular nature of the drug resistance factors of the Enterobacteriaceae. J. Bact. Biol. 17:376-393.
22. Morris, C.F., H. Hashimoto, S. Mickel, and R. H. Rownd (1974) Round of replication mutant of a drug resistance factor. J. Bact. 118:855-866.
23. Bolivar, F., R.L. Rodriquez, P.J. Green, M.C. Betlach, H.L. Heynecker, H.W. Boyer, J.H. Crosa, and S. Falkow (1977) Construction and characterization of new cloning vehicles. II. A multi-purpose cloning system. Gene 2:95-113.
24. Vieira, J., and J. Messing (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.
25. Messing, J., and J. Vieira (1982) A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19:269-276.
26. Forchhammer, J., E.N. Jackson, and C. Yanofsky (1972) Different half-lives of messenger RNA corresponding to different segments of the tryptophan operon of Escherichia coli. J. Mol. Biol. 71: 687-699.
27. Gillespie, D., and S. Spieglman (1965) A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12:829-842

. Shaw, W.V. (1975) Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. 43:737-755.
. Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Press, Cold Spring Harbor, New York.
. Tinoco, I.P., N. Borer, B. Dengler, M.D. Levine, O.C. Uhlenbeck, D.M. Crothers, and D. Gralla (1973) Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. 246:40-41.
. von Hippel, P.H., S.C. Kowalcyzkowski, N. Lonberg, J.W. Newport, and L.S. Paul 91982) Autoregulation of gene expression. Quantitative evaluation of the expression and function of the bacteriophage T4 gene 32 (single-stranded DNA binding) protein system. J. Mol. Biol. 162:795-818.
2. Shine, J., and L. Dalgarno (1974) The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci., USA 71:1342-1346.
3. Horinouchi, S., and B. Weisblum (1982) Nucleotide sequence and functional map of PE194, a plasmid that specifies inducible chloramphenicol resistance. J. Bact. 150:815-825.
4. Pritchard, R.H., P.T. Barth, and J. Collins (1969) Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19:263-297.
5. Jacob, F., S. Brenner, and F. Cuzin (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329-348.
6. Watson, J.D. (1976) Molecular Biology of the Gene, 3rd Edition. W.A. Benjamin, Inc., Reading, Massachusetts, p. 69.
7. Zuker, M., and P. Steigler (1981) Optimal computer folding of large RNA sequences using thermodynamics and auxillary information. Nucleic Acids Res. 98:133-148.
8. Salser, W. (1977) Globin mRNA sequences: Analysis of base pairing and evolutionary implications. Cold Spring Harbor Symp. Quant. Biol. 42:985-1102.

P1 PLASMID MAINTENANCE: A PARADIGM OF PRECISE CONTROL

Dhruba K. Chattoraj, Ann L. Abeles,
and Michael B. Yarmolinsky

Laboratory of Genetics and Recombinant DNA
LBI-Basic Research Program
NCI-Frederick Cancer Research Facility
Frederick, Maryland 21701

OVERVIEW

Certain bacterial plasmids, P1 prophage among them, are stably inherited despite their very low copy number (59). In each lysogenic cell, the mechanisms that guarantee a P1 replication prior to cell division and an equipartition of the daughter plasmids at cell division operate with error frequencies comparable to rates of mutation (7,61). In reviewing what we know about how such fidelity is achieved, this article will concentrate on plasmid replication and its control; the accompanying one by Austin and Abeles will consider partitioning.

A number of plasmid-encoded functions that contribute to stable plasmid maintenance have been distinguished. Discrete functions, however, are not necessarily the work of physically separable determinants. A replication origin may be internal to an initiator gene, as in λdv (77) or pT181 (34). An incompatibility determinant may be coincident with the determinant of its target, as in colE1 (76) or R1 (43). For several years, the hypothesis was widely accepted that the origin of plasmid replication and the site of partition are, in general, one and the same (32). We are fortunate that in the case of P1 it has been possible to dissect the determinants of the principal plasmid maintenance functions from each other and study them

By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article.

n isolation as well as variously combined. Dissection has revealed n origin of replication (oriR) and its associated specific initiaor (RepA) and control element (incA) (1,6); a partitioning apparaus that consists of a centromere analog with which an incompatibilty element (incB) is associated and 2 proteins (ParA and ParB) imlicated in its function (2,4); a site (loxP) (28) at which a proein (Cre) (2) efficiently resolves dimeric plasmids that might therwise interfere with plasmid equipartitioning (7,71); very likey, a mechanism that delays cell division when the plasmid copy numer falls below a critical threshold value (see later); and at least ne alternative origin under separate control (oriL) (70). The eleents of P1 mentioned above, partition elements aside, will be conidered below.

Our understanding of P1 maintenance has been aided by comparaive studies. For example, the concept of independent replication nd partition modules, made explicit with the demonstration of the unctionality of experimentally rearranged elements from the unreated plasmids P1 and F (3), had been adumbrated by comparison of he closely related plasmid prophages P1 and P7. Much of the parition regions of these largely homologous members of incompatibilty group Y appears to lie within a 2.5-kb region of P1/P7 nonhomolgy (29,83,84).

In addition to coliphages P1 and P7, the members of incompatiility group Y (26) include the closely related plasmid p15B 26,30), presumably a defective prophage; pIP231, an H_2S-producing lasmid (14); and the Salmonella phage j2 (52). P1 shows extensive ase-sequence homology, at least in the ori-repA-incA region, with lasmid Rts1, a member of incompatibility group T (35). Plasmids hat do not have a DNA sequence homology to P1 may, nevertheless, xhibit evidence of relatedness. The most striking example is the ertility factor F. Over those regions of F and P1 for which DNA equence comparisons have been made by computer analysis, hardly any ignificant homology is apparent (1), but in overall organization, he 2 plasmids are seen to exhibit a remarkable similarity (Fig. 1). Ve shall have more than one occasion to refer to implications of his relationship.

An aspect of plasmid replication that has been more thoroughly studied in F than in P1 is worth mentioning at the outset because of its importance; that is the manner of restoration of normal plasmid copy number in response to the introduction of excess copies of the lasmid. The added copies cause a total inhibition of plasmid replication until such time as cell division has reduced the plasmid copy number to its normal level (79). A very different restoration kinetics is characteristic of R1 (23). We assume that the manner by which P1 restores its copy number is essentially similar to that which has been reported for F.

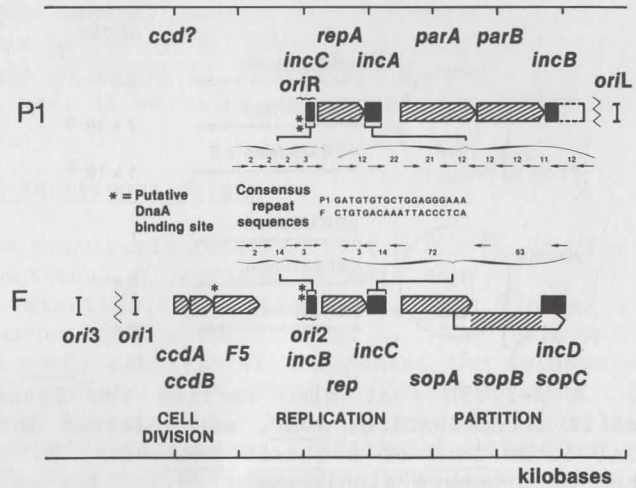


Fig. 1. The shaded arrows represent open reading frames for proteins that have been identified; black boxes represent segments that can exert incompatibility. Small arrows indicate the arrangements of the 19-bp repeat sequences. The distance between oriR and oriL is 10 ± 2 kb (70). The P1 coordinates of the replication region are from (1), and of the partition region from (5). The F coordinates of the cell division region are from (11,56), of the replication region are from (40,42) and of the partition region are from (57). There is evidence that ccdA and ccdB may each produce 2 small proteins (11). The pair of asterisks represent the sequence TTATCCACATTATCCAC in oriR of P1 and GTTATCCACTTATCCACG in ori2 of F; the single asterisk just within the putative open reading frame of the F5 protein represents the sequence GTTATACACAG (50).

ORIGINS

A Choice of Origins

Two separate and independent plasmid replicons have been isolated from a region of P1 DNA that represents about one-eighth of the genome and that contains, between them, the partition module. Because these replicons were detected in λ clones of rightward and leftward extensions of P1 fragment EcoRI-5, the original hybrids were designated λ-P1:5R and λP1:5L (70). The R and L replicons are subject to different controls. As will be discussed in a separate section, the R replicon is under control of the adjacent incA element, the determinant of group Y incompatibility; the L replicon is not (Ref. 73, N. Sternberg, unpub. data). The existence of an incA-insensitive plasmid replicon in a complete but chromosomally integrated P1 is indicated by the experiments of Fig. 2. Plasmid rep-

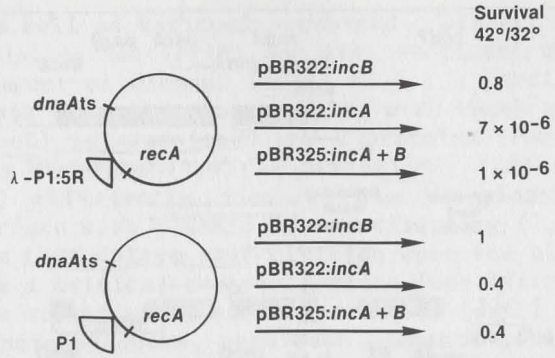


Fig. 2. Top. A λ-P1:5R that also carries the locus of site-specific recombination, loxP, was inserted into the bacterial locus loxB of a recA56 dnaA46(ts) E. coli by the method of prophage displacement (72). Bottom. P1Cm was similarly integrated.

The bacteria were transformed with the indicated pBR322 derivatives, spread on nutrient agar, and incubated 32°C and 42°C to determine the surviving fraction. Interference with survival at 42°C by an introduced inc element is taken as measure of interference with replication initiated from the P1 DNA.

lication initiating from λ-P1:5R or from P1 is seen to permit survival at 42°C of a dnaAts host into which these plasmids have been suitably integrated. Although inhibition of λ-P1:5R replication can be accomplished by additional copies of a cloned incA element, they are insufficient to inhibit markedly reduced plasmid replication initiated from P1. Preliminary experiments suggest that components of the P1 immunity system are required in addition to incA if P1-mediated plasmid replication is to be effectively blocked (D. Chattoraj and N. Sternberg, unpub. data).

The sensitivity of free P1 to destabilization by incA suggests that, during normal maintenance conditions, P1 is driven by the R replicon predominantly. Any alternative replicon of P1, if functional in the prophage state, is therefore insufficiently active to keep pace with cell division. However, an alternative replicon might be important in certain bacterial hosts other than E. coli or quite possibly during the establishment of lysogeny.

Alternative origins are not uncommon in bacterial plasmids. For example, a cluster of 3 origins has been noted in F (19,42,75). The central one of the 3, ori-1, is not expressed unless the nearby ori-2 is present, whereas the functioning of the ori-2 and ori-3 are independent of the presence of ori-1. The origin of the P1 R replicon, to which we have devoted most of our attention, appears to be analogous to ori-2 of F (Fig. 1). It remains to be determined whether there exist in P1 other origins with properties similar to those of F, and, if so, what advantages this extravagance might confer.

An Isolated Functional Origin

We have previously reported that a 2.1-kb P1 DNA segment determines a functional R replicon of unit copy (1). This mini-P1 includes the essential replication gene, repA flanked by the sets of 19-bp sequences indicated in Fig. 1. The set upstream of repA (designated incC) consists of 5 repeats; the downstream set (designated incA) consists of 9 repeats. Deletions that reduce the segment to 1.5 kb have recently been introduced without significant effect on plasmid replication. Additional deletions that eliminate all but ine and one-half repeats from incA result in a plasmid (pALA317) with elevated copy number (D. Chattoraj, K. Cordes, and A. Abeles, submitted for publication). In pALA317, there is only 1.2 kb of P1 DNA. Recently, it has been possible to dissect further this mini-P1 into a repA gene (without its own promoter) and a 245-bp region upstream of repA that is capable of acting as an origin when supplied in trans with a source of RepA protein. The construct pALA159 is essentially a P1 origin that depends entirely on the presence of pALA69, which furnishes RepA protein to it. The structures are given in Fig. 3, and the DNA sequence of the 245 bp of P1 present in pALA159 is shown in capital letters in Fig. 4. How much of this DNA is essential to origin function remains to be seen. Some hints are provided below.

Comparisons Among ori Sequences

A comparison of the 245-bp origin region of P1 with several other plasmid origins reveals a conserved sequence, GTTATCCACAGG, that, with minor variations, has been recognized as occurring 4 times in oriC of enterobacteria that belong to various genera (85) and once in the promoter area of a gene adjacent to oriC in Escherichia coli (24,25), as well as between the 2 promoters of the E. coli dnaA gene (24) and near the ends of the Tn5 (33). This sequence is generally found in association with several GATC sequences. In F, as in P1, variants of this sequence occur as a tandem pair (Fig. 4). An additional copy occurs near the beginning of the open reading frame (50) for the F5 protein of F (Fig. 1). Those that occur in oriC of E. coli bind dnaA protein specifically (20). Why dnaA protein binding sites should be conserved in plasmids such as P1, F, and pBR322 (46), that appear independent of dnaA, remains to be determined. Localized mutagenesis may be expected to answer this question.

We have included in Fig. 4 the DNA sequence of a primosome assembly site in F that has been identified as a segment of the oriS

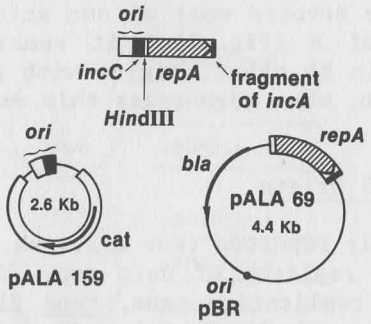


Fig. 3. The linear P1 fragment of the diagram carries a functional origin, repA, and 1-1/2 of the 9 repeat sequences of incA. The P1 DNA in pALA159 and pALA69 was derived from the linear fragment by HindIII cleavage at a site that borders on incC. Genes cat and bla determine chloramphenicol acetyl transferase and β-lactamase, respectively. Transcription of the repA gene of pALA69 is from an unidentified pBR322 promoter. The RepA protein furnished by pALA69 permits replication of pALA159. This complementation permits the 245-bp ori region to establish a copy number similar to that of plasmids in which ori and repA have not been separated.

region that might be essential for initiation. A scan of the 245-bp origin region of P1 reveals no analogous site, nor do we note such a site in P1 beyond the minimal origin within about 100 bp from the region of local P1/F homology described above. (In Fig. 4, P1 DNA exterior to the 245-bp region is distinguished by lower case letters.) It will, therefore, be of some interest to determine whether the primosome binding site plays a significant role in replication initiated at oriS. Paradoxically, a promising candidate for an essential site is present in only 1 of 2 similarly organized replicons, whereas sites deemed probably superfluous for both are uniquely conserved.

The most striking feature of the origin region compared in Fig. 4 has yet to be mentioned. That is the presence of from 3 to 5 copies of closely spaced, 18- to 21-bp sequences. These replicon-specific repeated sequences are so distributed as to present similar domains on a given side of the DNA helix, suggesting that their role is in the interactive binding of a specific protein characteristic of each replicon. In each of the cases that have been tested, the isolated set of repeated sequences exerts group-specific imcompatibility when present on a high copy-number vector (P1: Ref. 1, D. Chattoraj, K. Cordes, and A. Abeles, submitted for publication; Rts1: Ref. 36; F: Ref. 75,78; pSC101: Ref. 44). This incompatibility is most simply interpreted as a consequence of competition for a


```
P1      cattcctgtacgagggcgatgaccttaaatgctatgaacatggtcttctcatcgaaagcgtagaataatacgactccctTCCAACCGGCTACGTTGGCCGGTTTTTCAC
Rts1    TAGTTCCGGGAACCGTTGTCGGGTAATGAATGGGTTTACCTTTCATTGCCCGGCAGCGGTTGGACAAGCGTAGCGCGTCAGATATTCCTCCACATTACCATCATTTAAA
F       GCTCGCCGCAGTCGAGCGACGGCGAAGCCCTCGAGTGAGCGAGGAAGCACCAGGGAACAGCACTTATATATTCTGCTTACACACGATGCCTGAAAAAACTTCCCTTGGG
pSC101  GCCCGAGCTTGCGAGGGTGCTACTTAAGCCTTTAGGGTTTTAAGGTCTGTTTTGTAGAGGAGCAAACAGCGTTTGCGACATCCTTTTGTAATACTGCGGAACTGACTAA

P1      TTATCCACATTATCCACTGGATAGATCCAATAATCAGGTCCATACAGATCCCAATTAGATCCATATAGATCCCTGATCGTTGCAGGCCGCGCCACGTCTGGCTTAGAAGTG
Rts1    GTTATCCACATATCCACCGTGTAGATCCAATAATAGATCCATAGAGAGATCCAGATAAAACCAAAAAGATCCCCGTGGTCTGTAGCCTTACGCCACAAGGCTTACAACGTT
F       GTTATCCACTTATCCACGGGGATATTTTTATAATTATTTTTTTTATAGTTTTTAGATCTTCTTTTTTTAGAGCGCCTTGTAGGCCTTTATCCATGCTGGTTCTAGAGAAGG
pSC101  AGTAGTGAGTTATACACAGGGCTGGGATCTATTCTTTTTATCTTTTTTTATTCTTTATTCTATAAATTATAACCACTTGAATATAAACAAAAAAAACACACAAAGGTCTAG

P1      ATCGCGATGTGTGCTGGAGGGAAAACGATGTGTGCTGGAGGGATAAAAATGTGTGCTGACGGGTTGCTAATGTGTGCTGGCGGGATATAGGATGTGTGTTGACGGGAAAGC
Rts1    TTCGGTGTGTGCTGAGGGGAAAAAGGTGTGTGCTGAGGGGAAGAAAGTGTGTGTTACGGGGATTTGGGTGTGTGCTGAGGGGAAAAAAGGTGGGCGTCACGGGGAAAATGT
F       GTTGTGACAAATTGCCCTTTCAGTGTGACAAATCACCCTCAAAATGACAGTCCTGTCTGTGACAAATTGCCCTTAACCCTGTGACAAATTGCCCTCAGAAGAAGCTGTTTT
pSC101  GGAATTTACAGAGGGTCTAGCAGAATTTACAAGTTTTCCAGCAAAGGTCTAGCAGAATTTACAGATACCCACAACTCAAAGGAAAAGGACTAGTAATTATCATTGACTAGC
```

Fig. 4. The sequence of the 245-bp origin region of P1 is in capitals. Major repeat sequences are indicated by arrows, putative DnaA protein binding sites (20) by solid underlining, GATC sequences by overlining, and regions of homology to a primosome loading site in colE1 (31) by dashed lines. The pSC101 sequence given contains a region with some homology to F (marked by dashed lines and dots). The sequence of P1 is taken from (1), of Rts1 from (35), of F from (53), and of pSC101 from (16). See Fig. 5 for continuation of sequences.

limited supply of the essential initiator that the replicon determines. Both in vivo and in vitro experiments to be discussed below support this view.

Transcription Initiated in the Origin Region under RepA Control

Transcription initiated from the 245-bp origin region of P1 has been detected by galK operon fusion (Tab. 1). Transcription proceeds in both directions, although with unequal intensities. Both the strong promoter (prepA), which is pointed towards repA, and the weak promoter (pI), which is oppositely oriented, are repressed by RepA protein. The extent of this repression is most dramatic when the RepA protein is supplied from repA cloned in the high-copy-number vector pST52 (Tab. 1, line 3). Even the low, presumably physiological, level of RepA furnished by λ-P1:5R reduced by more than 90% the galK expression that is dependent upon prepA (Tab. 1, line 4); RepA has a considerable, possibly comparable, effect on

transcription from pI, although the effect is difficult to measure accurately because the promoter is so weak.

These results lend support to our supposition that the repeat units here, as in the origin regions of other replicons (22,77,80), are binding sites for a replicon-specific initiator. The supposition is further strengthened by recent experiments that indicate that both promoters lie within a 135-bp fragment, 104 bp of which are occupied by the incC set of repeats (data not shown). The observation that the initiator represses 2 differently oriented promoters suggests the possibility that it has more than one role to play. We consider next what these roles may be.

Tab. 1. Effect of incA and repA on transcription from the P1 origin region. Operon fusions were made between the 245-bp origin fragment of P1 and the galK gene of plasmids pK06 and pK04 (48). In pK06 (data column 1), the orientation of the fragment with respect to galK is the same as its orientation with respect to repA in P1; in pK04 (data column 2), the orientation is reversed. The plasmids were used to transform a galK2 recA3 strain of E. coli and galactokinase units were measured according to Ref. 48. The denominators of the tabulated ratios, i.e., the basal enzyme levels in bacteria harboring pK06 or pK04, varied between 3.5 and 3.8 units in different experiments. The pST52 vector is a RSF1030 derivative (66). The repA103 mutation is an amber mutation described in (1).

	Ratio of galactokinase units with/without origin fragment	
Source of incA & repA	Orientation 1 (prepA)	Orientation 2 (pI)
pST52 (vector)	48	2.0
pST52 incA	43	2.1
pST52 repA	0.3	0.4
λ-P1:5RrepA+	1.7	0.4
λ-P1:5RrepA103	40	2

THE RepA INITIATOR

Role of RepA in Replication

The capacity of pALA69 to permit the replication of the isolated P1 origin in pALA159 (Fig. 3) is presumably conferred by the repA gene. Detectable RepA protein is made in maxicells from the replication region of P1 cloned into pBR322 (pALA33), although the amount of protein made is low (1). By placing the repA gene under the λp_L promoter, it has been possible to synthesize the protein in high yield. After successive purifications on columns of afigel blue and phosphocellulose, the protein is about 90% pure. The relative electrophoretic mobility of repA indicates a protein of about 32 kd, in good agreement with the value of 32,224 predicted from the 286-codon open reading frame (1). RepA is typical of several plasmid initiator proteins that have been characterized with respect to such features as size and net positive charge (Tab. 2).

We would like to know how plasmid copy number varies as a function of repA expression and whether RepA protein is rate-limiting for replication under normal maintenance conditions. The data available, although minimal, reveal that the relationship is not a simple monotonic one. A high copy-number plasmid (pALA162) that supplies RepA in large excess, owing to the presence of a strong bla-p2 promoter, blocks the replication of various mini-P1 plasmids including λ-P1:5R and an incA-deleted plasmid, pALA139. Insertion

Tab. 2. Comparison of plasmid-determined initiator proteins.

Plasmid	Initiator	Mol. Wt. (Kd)	Net Charge	Autoregulation	Refs.
P1	RepA	32	+14	Yes	(1)*
Rts1	RepA	33	+13	Not determined	(35)
F	Rep (or E)	29	+16	Yes	(40, 58, 65, 82)
pSC101	Rep	37	+13	Yes	(16, 81)
R6K	π	35	+11	Yes	(21, 39)
pT181	RepC	37.5	+10	Not determined	(55)
λdv	O	34	+ 9	(Yes)†	(17, 47)

* See Table 3

† Autoregulator gene cro in same operon as initiator gene O.

f a moderately effective transcription terminator downstream from he strong promoter relieves this inhibitory effect. We conclude hat RepA in considerable excess is directly inhibitory to RepA-ediated replication. P1 appears not to be unique in possessing an nitiator protein that can exert a negative as well as a positive ffect on the initiation of replication. Studies of copy-number mu-ations in the initiator proteins of F (58) and R6K (68) indicate a imilar complexity of functions. Inhibitory effects on replication f initiator overproduction have been noted in R6K (27) and pSC101 G. Churchward, P. Linder, X. Guixan, and L. Caro, submitted for ublication).

Indirect evidence that the normal concentration of initiator rotein is such as to limit, rather than saturate or inhibit, the eplication machinery will be presented in subsequent sections.

epA Regulation of repA Transcription

An autoregulatory role of RepA is shown with the aid of repA-acZ fusion (Tab. 3). When RepA is furnished from a high-copy-umber plasmid, synthesis of the fusion protein is completely eli-inated. The promoter that is affected is the prepA in incC that we reviously noted to be markedly repressed by RepA. There does not ppear to be a more proximal promoter that is sufficiently active to e responsible for significant repA expression. By appropriate in-ertion of an efficient terminator, repA expression from a high opy-number plasmid could be made to depend solely on whatever pro-oters exist in the P1 DNA between incC and repA. The resultant lasmid failed to permit maintenance of the isolated origin plasmid, ALA159. Normal transcription of repA must, therefore, depend upon he highly RepA-sensitive promoter, prepA, that is in the set of ncC repeats.

The organization of P1 and F are not the same in the region be-ween the clustered origin repeats and the start of the initiator ene (Fig. 5). The transcription of the initiator gene of F has een found to originate primarily from a promoter in this region. he initiator is also autoregulated, but in this case, the proposed utoregulatory binding site, although recognizably related to the epeat sequences, is separated from the cluster (65). A similar eparation has been noted in R6K (69) and pSC101 (16). The auto-egulatory binding site is also significantly different in sequence rom any of the clustered sites. These structural differences are ikely to impart a different form to the dose-response curves for he activity of the initiator in replication and in the regulation f its own synthesis.

One implication of this statement is that it might be possible o mutationally alter the autoregulated initiator protein so as to ifferentially affect 1 of its 2 roles. A mutation in the initiator

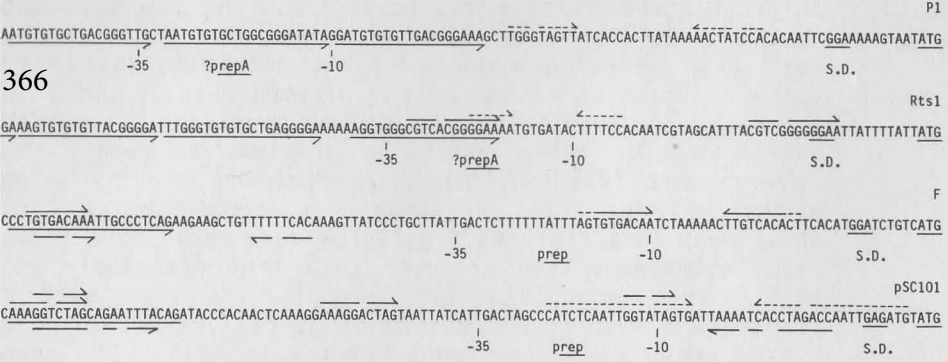
Tab. 3. Autoregulation of repA transcription. Fusions were made that join in frame the first 66 codons of repA DNA to the lacZ gene (missing codons 1-8) of pMLB1034 (64). The prepA- containing P1 DNA of the plasmid used in obtaining the data of column 1 included 297 bp upstream of repA (the 245-bp origin region and 52 bp proximal to repA). The other insert (data of column 2) included only 56 bp upstream of repA. By providing a ribosome-binding site, this 56-bp insert allows β-galactosidase expression from a weak RepA-insensitive promoter (p?) that presumably lies within the vector itself. The plasmids were used to transform a Δlac recA56 strain of E. coli, and β-galactosidase units were measured according to (51). The basal enzyme level in bacteria harboring pMLB1034 was <1 unit.

	β-galactosidase units from repA-lacZ protein fusion	
Source of repA	prepA	p?
pST52 (vector)	2700	42
pST52 repA	< 1	43

that specifically diminishes its autoregulatory role would cause an increase in plasmid copy number, provided that replication was initiator-limited. At least some cop mutations that have been identified in P1 (9) and in F (58,63) have been localized to the respective initiator genes and possibly belong to this category.

Specific Binding of RepA to Repeat Sequences

The genetic arguments for RepA binding to the DNA repeats are supported by a direct physical demonstration of sequence-specific binding (Fig. 6). We look forward to obtaining quantitative data with which to answer questions about the stoichiometry and cooperativity of this binding. Cooperative interactions presumably lie at the heart of precise replication control.



ig. 5. Characteristic -10 and -35 sequences (60) of likely promoters for transcription of the initiator proteins of P1 and Rts1 are the choices of the authors based on a TARGSEARCH analysis (54). The corresponding choices for F and pSC101 are those of (65) and (16), respectively. The P1 sequence is taken from (1), the Rts1 sequence from (35). Arrows without dashes represent major repeats of repeated segments thereof. Certain of these are postulated to be autoregulatory sites. Dashed arrows represent other (inverted) repeats.

EPLICATION CONTROL ELEMENT incA

ispensability of incA

In P1, Rts1, and F, the repeats that bind (or presumably bind) he plasmid-determined initiators are found flanking the respective nitiator genes. The downstream repeats are more numerous than the pstream repeats, are less densely distributed, and appear to play a ifferent role. Whereas the upstream repeats (of F, and presumably f P1) appear to be an essential component of the origin (M. outurier, pers. comm.), the downstream repeats are dispensable. limination from a mini-P1 of 7 1/2 of the 9 downstream (incA) reeats results in a 6-fold increase in plasmid copy number (D. hattoraj, K. Cordes, and A. Abeles, submitted for publication). In , the deletion of all repeat units downstream of the initiator gene as been achieved and increases the copy number about 6- to 8-fold 10,63,78). Loss of these repeats does not result in runaway replication.

Reinsertion of incA into a mini-P1 that has suffered a substanial deletion of incA repeats (pALA139) restores the plasmid to a

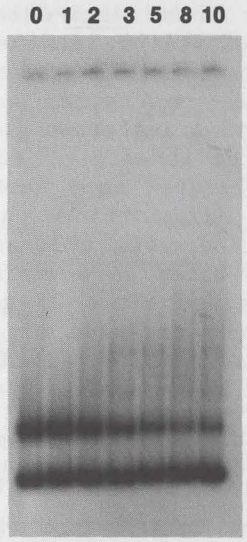


Fig. 6. In vitro binding of RepA protein to repeat sequences.

A fragment of DNA containing the 9 incA repeats and a control piece of pBR322 DNA were excised from a pBR322 clone carrying incA and end-labeled with ^{32}P dNTP. The fragments were then mixed with the indicated increasing amounts of purified RepA and electrophoresed in a 5% acrylamide gel. The autoradiogram shows that the incA fragment formed several slow-moving bands, apparently by complexing with the protein, whereas the mobility of the control fragment was not affected. Binding of RepA to incC has been demonstrated in similar experiments (data not shown).

low copy number. The incA can be variously positioned within the plasmid or placed in trans within a chromosomally integrated λ prophage. The effects on copy number of such reinsertions are comparable, at least to a first approximation. Therefore, neither the position nor the orientation of this set of repeats is of major significance in determining copy number. The results of these experiments also imply that the incA repeats are dispensable in the target replicon for sensitivity to the destabilizing action of incA (Tab. 4). The latter deduction is further strengthened by an experiment (not shown) in which a single copy of incA per bacterial chromosome destabilizes the isolated origin plasmid pALA159 when the source of RepA is from pALA69, a plasmid in which repA is transcribed from an unidentified vector promoter, rather than from a P1 promoter.

Tab. 4. Destabilization of an incA-deleted mini-P1, pALA139, by incA in trans. pALA139 retains only 1 1/2 incA repeats (as little of incA as is present in the chimera of line 3), a functional repA gene, a P1 origin, and a chloramphenicol acetyl transferase gene. The plasmid is stably maintained in the absence of equipartition functions because of its elevated copy number relative to its incA$^+$ parent. It can be maintained in the presence of 1 copy of incA by selection with chloramphenicol. The tabulated percentages are based on a minimum of 64 colonies tested for drug resistance after 25 generations of growth with selection for the presence of pBR322 alone or λ alone. The bacterium was recA3 strain N100.

Vector and incA repeats	Percent bacteria retaining pALA139 after 25 generations
1) pBR322: all nine	<2
2) pBR322: part of #7, #8, #9	<2
3) pBR322: part of #8, #9	94
4) pBR322: none	>99
5) λ prophage: all nine	<1
6) λ prophage: none	>99

We conclude that the incA present in unit-copy mini-P1 is dispensable for incA sensitivity, as it is for the capacity to replicate. It is an element in replication control that is capable of inhibiting a P1 replicon when present as a single extra copy.

Replication Control Exerted by Subsets of incA

To determine the mechanism(s) by which incA exerts its control, we have asked whether destruction of the integrity of incA influences its activity. The efficacies of subsets of the incA repeats cloned into pBR322 are presented in Tab. 4 and 5. Like the complete set of 9 repeats, each set of 3 repeats tested causes more than 98% loss of λ-P1:5R within 25 generations. A clone of less than 2 repeats failed to exert significant incompatibility by this criterion.

Tab. 5. Destabilization of the mini-P1 λ-P1:5R by fragments of incA. The experiments are similar to those of Tab. 4 except that loss of the mini-P1 was followed by loss of the λ immunity marker.

incA repeats carried by pBR322 vector	Percent bacteria retaining λ-P1:5R after 25 generations
1) all nine	<3
2) #1, #2, #3	<1
3) #4, #5, #6	<1
4) #7, #8, #9	<3
5) part of #7, #8, #9	3
6) part of #8, #9	96
7) none	>99

By a less sensitive test, based on dnaAts integrative suppression, none of the set of 3 repeats was effective in expressing incompatibility, but 2 different sets of 6 repeats were as effective as intact incA (data not shown). These results demonstrate that no unique site within incA is required for incA activity (Tab. 4 and 5). Since even 2 1/2 repeats exert marked incompatibility when present in high copy number, we consider it unlikely that incA encodes small RNA or oligopeptide mediators of incompatibility. However, we do not imply that the repeats act independently of one another.

Relief by RepA of Replication Block by incA Repeats

There is a simple way to explain how the subsets of incA can cause specific replication inhibition. We suggest that the DNA repeats inhibit initiator function by uniting with the specific initiator. Provided that an appreciable fraction of the initiator can be removed by binding to incA, nothing more elaborate than a mass action effect need be postulated. According to this hypothesis, incompatibility expression by incA should be relieved by an increased supply of RepA. The results presented in Tab. 6 show that a source of RepA overcomes the blockage by incA of integrative dnaAts suppression. This relief can be overcome, in turn, by additional copies of incA. We have already seen in Tab. 1 that incA by itself does not affect transcription from the origin. Both these experi-

Tab. 6. Replication block by *incA* relieved by *repA*. At least 2 λ *incA* prophages are required to block integrative suppression of the *dnaAts* defect afforded by the integrated mini-P1, λ-P1:5R. One λ*incA* prophage is insufficient. RepA is overproduced from the pBR322 *bla-p2* attenuated by a transcription terminator promoter in the plasmid (pA1A176) of lines 5 and 6. The relief afforded by this overproduction of RepA, in turn, overcome by furnishing *incA* from the high-copy-number plasmid pST52 *incA*. A small fragment of *incA* is associated with the cloned *repA* gene and is the same fragment that is shown in line 3 of Tab. 4 to exert negligible incompatibility even when in high copy number. The pBR322 vector used here contained an inserted chloramphenicol acetyl transferase gene.

Episomes carried by *recA56 dnaA46(ts) E. coli*			Surviving fraction
Integrated at *loxB*	Prophages at *attλ*	Plasmids	42°/32°
1) none	none	none	4×10^{-6}
2) λ-P1:5R	none	none	1
3) λ-P1:5R	(λ*incA*)	none	5×10^{-4}
4) λ-P1:5R	(λ*incA*)	pBR322	5×10^{-4}
5) λ-P1:5R	(λ*incA*)	pBR322 *repA*	1
6) λ-P1:5R	(λ*incA*)	pBR322 *repA*; pST52 *incA*	2×10^{-6}

ments support the contention that *incA* controls copy number by sequestering the rate-limiting RepA protein.

A direct demonstration that *incA* binds *repA* protein is shown in Fig. 6. Quantitative measurements of affinities of the initiator protein for the repeats before and after the initiator gene have not yet been made. We expect that the *incA* repeats are loaded with RepA before the putative site that triggers replication is filled. We also expect that the relative affinities of RepA for repeat groups

of various sizes are correlated with their relative strengths as incompatibility elements.

REPLICATION CONTROL

How are the Antagonistic Control Circuits to be Reconciled?

The arguments presented for a control of RepA concentration by autoregulation and by binding to *incA* may appear separately compelling, but these 2 possibilities are also mutually exclusive. A responsive autoregulatory circuit would nullify any attempt to regulate the concentration of free RepA by varying the concentration of RepA binding sites. If the 2 modes of control are to have any biological meaning, their operation must not be such that one control inevitably nullifies the other.

A reconciliation might be achieved if there existed a concentration range of RepA over which autoregulation is effective and a different concentration range over which *incA* is effective. Unfortunately, the evidence suggests that both kinds of controls operate at the same RepA concentration, i.e., when a single copy of a mini-P1 is present.

A more satisfactory resolution of the problem may be to invoke different kinetics for the 2 control circuits. The following formulation of the "over shoot model" is, in part, the fruit of discussions with Kurt Nordström and Robert Pritchard, among others, but the component ideas have originated independently in several laboratories. The model depends on the existence of a refractory period in the autoregulatory circuit that is due to the finite duration of transcription, translation, and message decay. Let us suppose that the refractory period in the autoregulatory circuit is sufficiently long that, after the *incA* repeats have filled with initiator protein, free initiator can transiently accumulate up to a threshold concentration or amount necessary for replication to be initiated. Although replication will double the initiator gene dosage, the concentration of free initiator will fall sharply rather than rise. At the time of replication, the delayed autoregulatory shut-off of initiator synthesis will have occurred and, as has been proposed for the analogous case of F (78), existing initiator molecules will bind to the newly created repeats. Until these repeats fill and free initiator accumulates again, there will be no reinitiation of replication. In this model, there is a hierarchy of affinities for initiator protein. The autoregulatory site will have the highest affinity, the copy-number control repeats a lesser affinity, and the site(s) that triggers replication the lowest. The model also assumes a concentration of free initiator protein sufficiently low that titration by a few repeats can be significant. Titration must

lso be slow enough to account for the observed interreplication imes.

An alternative resolution of the problem can be made if the utoregulator and the initiator of replication are assumed to be ifferent molecular species that are determined by the same operon. model of this kind was proposed for E. coli chromosome replication n 1973 (67) and was shown to operate in the case of λdv, which is ontrolled by the cotranscribed cro autoregulator and the O initiaor (47). The applicability of this model to F is suggested by J.D. rawick and B.C. Kline (submitted for publication; and Ref. 38). hese authors propose, for simplicity, that the initiator is a odified form of the autoregulator, perhaps derived from it by ligomerization. If the rate of this modification is so slow that nly a very low concentration of initiator forms, its titration by epeats can significantly alter the availability of initiator at the ite that triggers replication. Initiator titration need not affect utoregulation. The relative concentration of autoregulator is not pecified by the model and the affinity of the autoregulatory site or the autoregulator need not be particularly high. Although this nd the preceding model have certain features in common, there are estable differences between them. Both these models adequately ccount for the abrupt adjustment of plasmid replication rates that s characteristic of F (79) and presumably of P1.

Autoregulated initiators are characteristic of a number of diferent plasmids (Tab. 2). A rationale for this autoregulation is pparent. In general, replacement of an autoregulated rate-limiting initiator by a constitutive one would cause the plasmid copy number o increase out of control or, in some cases, only to the point here further initiator synthesis would inhibit plasmid replication.

The separate set of copy-control repeats that are found in P1, tsl, and F play a less obvious role in plasmid maintenance. Runway replication does not occur when these repeats are deleted, lthough, as previously mentioned, elevated copy numbers do result. o appreciate the possible utility of these repeats in plasmid mainenance, we ask what disadvantage might their loss confer on a lasmid that correspondingly reduced the synthesis (or efficacy) of he initiator protein to compensate for its increased availability or triggering replication. Such a compensated loss would leave the verage plasmid copy number unaltered. However, the longer the set f binding sites, the more reproducible will be the length of the eriod needed for initiator resynthesis that will follow each DNA uplication. Without incA, the refractory periods between succesive rounds of replication would be more variable. A broadened istribution of plasmid copy numbers in the bacterial population ould cause an increased proportion of the bacterial population to e disadvantaged in the sense of carrying an excessive plasmid urden or none at all.

A way of formally analyzing the situation has been suggested to us by Dr. Luud Koppes (University of Uppsala). He proposes that the interval between successive replications can be regarded as the waiting time for successive random associations of initiator with a particular number of repeats that must be filled before DNA synthesis initiates. It is assumed that the occupation of a particular repeat unit at the origin region triggers the initiation of DNA synthesis and occurs only after an orderly filling of the other repeats. Koppes calculates, in an idealized case, that the coefficient of variation of the interreplication time is proportional to the reciprocal of the square root of the total number of repeats. The important conclusion is that the larger the number of repeats, the smaller the fluctuation.

It is not excluded that an adequate explanation of P1 plasmid maintenance will require the invocation of an additional control loop whose existence has been overlooked. Evidence for the existence of 1 additional stabilizing circuit that we believe is present in intact P1, but not in the mini-P1 plasmids so far considered, will be presented next.

COORDINATION WITH CELL DIVISION

For a plasmid to be maintained as stably as is P1 or F, fluctuation in interreplication time that could result in plasmid loss cannot be tolerated. It is not surprising that a special mechanism should have evolved to ensure that emergency measures are invoked when there is an immediate threat of such loss.

Any of a variety of unit-copy plasmids that have been impaired in their capacity to replicate and then introduced into healthy bacteria cause a temporary cessation of cell division. The bacteria filament and subsequently exhibit other SOS responses as if they had been damaged themselves (8,12,13,18,41,45,62). These responses can be brought about by UV irradiation of the plasmid or by more specific means of reducing plasmid copy number. Repair of damage to the plasmid is favored, and cell division prior to repair is inhibited. The genetic basis for this coupling of cell division to the well-being of the plasmid has been determined in F (8,11,13,37,49,50,56). The location of the coordination with cell division (ccd) genes of F is shown in Fig. 1. The ccdB gene product(s) inhibits cell division; the ccdA gene product(s) normally prevents this inhibition but cannot do so at gene dosages that are so low as to present a threat to plasmid stability.

Although ccd genes have not been identified in P1, there are indications that a ccd function is determined within EcoRI-8 of P1, in a region situated similarly to that of F. EcoRI-8 is a 6-kb fragment that carries the P1 origin near its junction with EcoRI-5. Mini-P1 plasmids that carry EcoRI-8 have been shown to have the po-

tential for causing SOS induction (15), in contrast to the diminutive mini-P1 plasmids that we have studied (M. Yarmolinsky, unpub. data). In view of the several similarities between P1 and F that we have already noted, it would be surprising to find that both plasmids have adopted similar strategies for addicting the host to their presence.

CONCLUSIONS: THE CLEPSYDRA ANALOGY

The mechanisms of plasmid tenacity discussed in this and the accompanying article are of some antiquity because they appear to be common to diverse replicons that, by normal criteria, are nonhomologous. In several plasmids, a modular arrangement of DNA regions that are involved in replication and its control, coordination with cell division, partitioning, and dimer resolution enables us to perform a variety of mix-and-match experiments. Comparative studies make it clear that Nature was doing this long before our arrival on the scene. The process has been named reticulate (as opposed to arboreal) evolution.

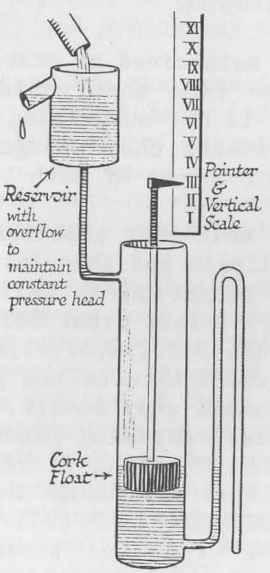


Fig. 7. The upper reservoir, with its means of maintaining a constant pressure head, resembles the autoregulated source of RepA. The lower vessel resembles the set of repeats that must be filled before a putative critical repeat can fill and trigger the sudden creation by replication of a set of empty repeats. Alterations in the length of the siphon tube correspond to alterations in the number of incA repeat sequences and affect the interval between cycles.

The P1 control circuit to which we have paid most attention involves the regulation of DNA synthesis by a stretch of DNA that acts as a sink or chambered reservoir for an essential initiator protein. We have suggested that filling the chambers triggers a round of DNA synthesis that generates a new empty reservoir. The action is much like that of a pipet washer that fills slowly up to the point where a siphon empties the vessel suddenly, whereupon it recommences the cycle. The principle was used more than 2,000 years ago in the construction of water clocks (clepsydras) (Fig. 7). In some clepsydras, constancy of the cycles was assured by having the reservoir filled from a tank that was maintained at the level of an overflow. This constant influx of water is analogous to a synthesis of initiator that, due to autoregulation, is at least partially buffered against changes in gene dosage. We would like to believe that the repeated DNA sequences and the control circuits associated with them form a molecular clock by which to regulate plasmid generation times.

We are attracted to plasmids as objects of study because they are dispensable elements. Ironically, we are most fascinated by the ingenuity of the mechanisms that make a resident plasmid almost impossible to disown, mechanisms that appear to be among Nature's early inventions. It is appropriate that one of these mechanisms may represent a molecular analog of an early human invention.

ACKNOWLEDGMENTS

We thank Martin Mulligan and William McClure of Carnegie-Mellon University for providing us with a TARGSEARCH computer analysis of potential promoters in the region upstream of the repA gene of P1; Bruce Kline, Luud Koppes, Kurt Nordström, and Robert Pritchard for helpful discussions of regulatory circuitry; and Julie Ratliff for cheerful preparation of the manuscript, sequences included.

Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-23909 with Litton Bionetics, Inc. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

1. Abeles, A.L., K.M. Snyder, and D.K. Chattoraj (1984) P1 plasmid replication: Replicon structure. J. Mol. Biol. 173:307-324.
2. Abremski, K., and R. Hoess (1983) Bacteriophage P1 site-specific recombination: Purification and properties of the Cre recombinase protein. J. Biol. Chem. 259:1509-1519.

3. Austin, S., and A. Abeles (1983) Partition of unit-copy miniplasmids to daughter cells. I. P1 and F miniplasmids contain discrete interchangeable sequences sufficient to promote equipartition. J. Mol. Biol. 173:353-372.
4. Austin, S., and A. Abeles (1983) Partition of unit-copy miniplasmids to daughter cells. II. The partition region of miniplasmid P1 encodes an essential protein and a centromere-like site at which it acts. J. Mol. Biol. 169:373-387.
5. Austin, S., and A. Abeles (1984) The partition functions of P1, P7 and F miniplasmids. This volume.
6. Austin, S., F. Hart, A. Abeles, and N. Sternberg (1982) Genetic and physical map of a P1 miniplasmid. J. Bact. 152:63-71.
7. Austin, S., M. Ziese, and N. Sternberg (1981) A novel role for site-specific recombination in maintenance of bacterial replicons. Cell 25:729-736.
8. Bailone, A., R. Devoret, A. Brandenburger, A. Lévine, M. Dutreix, and M. Pierre (1984) Aborted installation or maintenance of mini-F induces SOS functions. Abstract, this volume.
9. Baumstark, B.R., L. Lowery, and J.R. Scott (1984) Location by DNA sequence analysis of cop mutations affecting the number of plasmid copies of prophage P1. Mol. Gen. Genet. (in press).
10. Bergquist, P.L., R.A. Downard, P.A. Caughey, R.C. Gardner, and H.E.D. Lane (1981) Analysis of mini-F plasmid replication by transposition mutagenesis. J. Bact. 147:888-899.
11. Bex, F., H. Karoui, L. Rokeach, P. Drèze, L. Garcia, and M. Couturier (1983) Mini-F encoded proteins: Identification of a new 10.5 kilodalton species. EMBO J. 2:1853-1861.
12. Borek, E., and A. Ryan (1958) The transfer of irradiation-elicited induction in a lysogenic organism. Proc. Natl. Acad. Sci., USA 44:374-377.
13. Brandenburger, A., A. Bailone, A. Lévine, and R. Devoret (1984) The SOS signal produced by miscarriage of an intact mini-F plasmid. J. Mol. Biol. (in press).
14. Briaux S., G. Gerbaud, and A. Jaffé-Brachet (1979) Studies of a plasmid coding tetracycline resistance and hydrogen sulfide production incompatible with the prophage P1. Mol. Gen. Genet. 170:319-325.
15. Capage, M.A., and J.R. Scott (1983) SOS induction by P1Km miniplasmids. J. Bact. 155:473-480.
16. Churchward, G., P. Linder, and L. Caro (1983) The nucleotide sequence of replication and maintenance functions encoded by plasmid pSC101. Nucleic Acids Res. 11:5645-5659.
17. Daniels, D.L., J.S. Schröeder, W. Szybalski, F. Sanger, A.R. Coulson, G.F. Huong, D.F. Hill, G.B. Petersen, and F.R. Blattner (1983) Complete annotated lambda sequence. In Lambda II, R.W. Hendrix, J.W. Roberts, F.W. Stahl and R.A. Weisberg, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 519-676.
18. Devoret, R., and J. George (1967) Induction indirecte du prophage λ par le rayonment untraviolet. Mutat. Res. 4:713-725.

19. Eichenlaub, R., D. Figurski, and D. Helinski (1977) Bidirectional replication from a unique origin in a mini-F plasmid. Proc. Natl. Acad. Sci., USA 74:1138-1141.
20. Fuller, R.S., and A. Kornberg (1983) Purified dnaA protein in initiation of replication at the Escherichia coli chromosomal origin of replication. Proc. Natl. Acad. Sci., USA 80:5817-5821.
21. Germino, J., and D. Bastia (1982) Primary structure of the replication initiation protein of plasmid R6K. Proc. Natl. Acad. Sci., USA 79:5474-5479.
22. Germino, J., and D. Bastia (1983) Interaction of the plasmid R6K-encoded replication initiator protein with its binding sites on DNA. Cell 34:125-134.
23. Gustafsson, P., and K. Nordström (1980) Control of replication of plasmid R1. Kinetics of replication in shifts between different copy number levels. J. Bact. 141:106-110.
24. Hansen, E.B., F.G. Hansen, and K. von Meyenburg (1982) The nucleotide sequence of the dnaA gene and the first part of the dnaN gene of Escherichia coli K-12. Nucl. Acids Res. 10:7373-7385.
25. Hansen, F.G., S. Koefoel, K. von Meyenburg, and T. Atlung (1981) In The Initiation of DNA Replication, ICN-UCLA Symposia on Molecular and Cellular Biology, D.S. Ray and C.F. Fox, eds. Academic Press, New York, Vol. 21, pp. 37-55.
26. Hedges, R.W., A.E. Jacob, P.T. Barth, and N.J. Grinter (1975) Compatibility properties of P1 and ΦAMP prophages. Mol. Gen. Genet. 141:263-267.
27. Helinski, D. (1984) This volume.
28. Hoess, R., M. Ziese, and N. Sternberg (1982) P1 site-specific recombination: Nucleotide sequences of the recombining sites. Proc. Natl. Acad. Sci., USA 79:3398-3402.
29. Iida, S., and W. Arber (1979) Multiple physical differences in the genome structure of functionally related bacteriophages P1 and P7. Mol. Gen. Genet. 173:249-261.
30. Ikeda, H., M. Inuzuka, and J-I. Tomizawa (1970) P1-like plasmid in Escherichia coli 15. J. Mol. Biol. 50:457-470.
31. Imber R., R.L. Low, and D.S. Ray (1983) Identification of a primosome assembly site in the region of the ori2 replication origin of the Escherichia coli mini-F plasmid. Proc. Natl. Acad. Sci., USA 80:7132-7136.
32. Jacob, F., S. Brenner, and F. Cuzin (1963) On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 20:329-348.
33. Johnson, R.C., and W.S. Reznikoff (1983) DNA sequences at the ends of transposon Tn5 required for transposition. Nature 304:280-282.
34. Kahn, S.A., G.K. Adler, and R.P. Novick (1982) Functional origin of replication of plasmid pT181 DNA is contained within a 168 base pair segment. Proc. Natl. Acad. Sci., USA 79:4580-4584.

35. Kamio, Y., A. Tabuchi, Y. Itoh, H. Katagiri, and Y. Terawaki (1984) Complete nucleotide sequence of mini-Rts1 and its copy mutant. J. Bact. 158:307-312.
36. Kamio, Y., and Y. Terawaki (1983) Nucleotide sequence of an incompatibility region of mini-Rts1 that contains five direct repeats. J. Bact. 155:1185-1191.
37. Karoui, H., F. Bex, P. Drèze, and M. Couturier (1983) Ham22, a mini-F mutation which is lethal to host cell and promotes recA-dependent induction of lambdoid prophage. EMBO J. 2:1863-1868.
38. Kline, B.C., and J.D. Trawick (1984) A proposed model for control of mini-F replication. Abstract, this volume.
39. Kolter, R., and D. Helinski (1982) Plasmid R6K DNA replication II. Direct nucleotide sequence repeats are required for an active γ-origin. J. Mol. Biol. 161:45-56.
40. Komai, N., T. Nishizawa, Y. Hayakawa, T. Murotsu, and K. Matsubara (1982) Detection and mapping of six mini F-encoded proteins by cloning analysis of dissected mini-F segments. Mol. Gen. Genet. 186:193-203.
41. Koyama, A.H., and T. Yura (1975) Plasmid mutations affecting self-maintenance and host growth in Escherichia coli. J. Bact. 122:80-88.
42. Lane, D., and R.C. Gardner (1979) Second EcoRI fragment of F capable of self-replication. J. Bact. 139:141-151.
43. Light, J., and S. Molin (1982) The sites of action of the two copy number control functions of plasmid R1. Mol. Gen. Genet. 187:486-493.
44. Linder, P., G. Churchward, and L. Caro (1983) Plasmid pSC101 replication mutants generated by insertion of the transposon Tn1000. J. Mol. Biol. 170:287-303.
45. MacQueen, H.A., and W.D. Donachie (1977) Intracellular localization and effects on cell division of a plasmid blocked in deoxyribonucleic acid replication. J. Bact. 132:392-397.
46. Sutcliffe, J.G. (1978) The complete nucleotide sequence of the Escherichia coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43:77-90/
47. Matsubara, K. (1981) Replication control system in lambda dv. Plasmid 5:32-52.
48. McKenney, K., H. Shimatake, D. Court, U. Schmeissner, C. Brady, and M. Rosenberg (1981) In Gene Amplification and Analysis, J.G. Chirikjian and T.S. Papas, eds. Elsevier-North Holland, Amsterdam, Vol. 2, pp. 384-417.
49. Miki, T., Z-T. Chang, and T. Horiuchi (1984) Control of cell division by sex factor F in Escherichia coli II. Identification of genes for inhibitor proteins and trigger protein on the 42.84-43.6 F segment. J. Mol. Biol. 174 (in press).
50. Miki, T., K. Yoshioka, and T. Horiuchi (1984) Control of cell division by sex factor F in Escherichia coli. I. The 42.84-43.6 F segment couples cell division of the host bacteria with replication of plasmid DNA. J. Mol. Biol. 174 (in press).
51. Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

52. Mise, K., Y. Yoshida, and M. Kawai (1983) Generalized transduction between Salmonella typhi and Salmonella typhimurium by phage j2 and characterization of the j2 plasmid in Escherichia coli. J. Gen. Microbiol. 129:3395-3400.
53. Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami (1981) Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene 15:257-271.
54. Mulligan, M.E., D.K. Hawley, R. Entriken, and W.R. McClure (1984) Escherichia coli promoter sequences predict in vitro RNA polymerase selectivity. Nucl. Acids Res., (in press).
55. Novick, R.P., G.K. Adler, S. Majumder, S.A. Khan, S. Carleton, W.D. Rosenblum, and S. Iordanescu (1982) Coding sequence for the pT181 repC product: A plasmid-coded protein uniquely required replication. Proc. Natl. Acad. Sci. 79:4108-4112.
56. Ogura, T., and S. Hiraga (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci., USA 80:4784-4788.
57. Ogura, T., S. Hiraga (1983) Partition mechanism of F plasmid: 2 plasmid gene-encoded product and a cis-acting region are involved in partition. Cell 32:351-360.
58. Piérard, P., J. Janssens, L. Garcia, P. Drèze, M. Couturier, and F. Bex (1984) Mini-F copy number mutations affecting the primary structure of the E protein. Arch. Int. de Phys. et de Bioch. 92:B52-B53.
59. Prentki, P., M. Chandler, and L. Caro (1977) Replication of the prophage P1 during the cell cycle of Escherichia coli. Mol. Gen. Genet. 152:71-76.
60. Rosenberg, M., and D. Court (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. 13:319-353.
61. Rosner, J.L. (1972) Formation, induction and curing of bacteriophage P1 lysogens. Virology 48:679-689.
62. Rosner, J.L., L.R. Kass, and M.B. Yarmolinsky (1968) Parallel behavior of F and P1 in causing indirect induction of lysogenic bacteria. Cold Spring Harbor Symp. Quant. Biol. 33:784-789.
63. Seelke, R.W., B.C. Kline, J.D. Trawick, and G.D. Ritts (1982) Genetic studies of F plasmid maintenance genes involved in copy number control, incompatibility and partitioning. Plasmid 7:163-179.
64. Silhavy, T.J., M.L. Berman, and L.W. Enquist (1984) Experiments with Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
65. Søgaard-Anderson, L., L.A. Rokeach, and S. Molin (1984) Regulated expression of a gene important for replication of plasmid F in E. coli. EMBO J. 3:257-262.
66. Som, T., and J-I. Tomizawa (1982) Origin of replication of Escherichia coli plasmid RSF1030. Mol. Gen. Genet. 187:375-383.
67. Sompayrac, L., and O. Maaløe (1973) Autorepressor model for control DNA replication. Nature New Biol. 241:133-135.

68. Stalker, D.M., R. Kolter, and D.R. Helinski (1982) Plasmid R6K replication I. Complete nucleotide sequence of an autonomously replicating segment. J. Mol. Biol. 161:33-43.
69. Stalker, D.M., M. Filutowicz, and D.R. Helinski (1983) Release of initiation control by a mutational alteration in the R6K π protein required for plasmid replication. Proc. Natl. Acad. Sci., USA 80:5500-5504.
70. Sternberg, N., and S. Austin (1983) Isolation and characterization of P1 minireplicons, λ-P1:5R and λ-P1:5L. J. Bact. 153:800-812.
71. Sternberg, N., and D. Hamilton (1981) Bacteriophage P1 site-specific recombination. I. Recombination between 2 loxP sites. J. Mol. Biol. 150:467-486.
72. Sternberg, N., D. Hamilton, and R. Hoess (1981) P1 site-specific recombination. II. Recombination between loxP and the bacterial chromosome. J. Mol. Biol. 150:487-507.
73. Sternberg, N., and R. Hoess (1983) The molecular genetics of bacteriophage P1. Annu. Rev. Genet. 17:123-154.
74. Tolun, A., and D. Helinski (1981) Direct repeats of the F plasmid incC region express F incompatibility. Cell 24:687-694.
75. Tolun A., and D. Helinski (1982) Separation of the minimal replication region of the F plasmid into a replication origin segment and a transacting segment. Mol. Gen. Genet. 186:372-377.
76. Tomizawa, J-I., and T. Itoh (1981) Plasmid colE1 incompatibility determined by interaction of RNAI with primer transcript. Proc. Natl. Acad. Sci., USA 78:6096-6100.
77. Tsurimoto, T., and K. Matsubara (1981) Purified bacteriophage λO protein binds to four repeating sequences at the λ replication origin. Nucl. Acids Res. 9:1789-1799.
78. Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsubara (1983) Role of 9 repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bact. 155:337-344.
79. Tsutsui, H., and K. Matsubara (1981) Replication control and switch-off function as observed with a mini-F factor plasmid. J. Bact. 147:509-516.
80. Vocke, C., and D. Bastia (1983) DNA-protein interaction at the origin of DNA replication of the plasmid pSC101. Cell 35:495-502.
81. Vocke, C., and D. Bastia (1983) Primary structure of the essential replicon of the plasmid pSC101. Proc. Natl. Acad. Sci., USA 80:6557-6561.
82. Wehlmann, H., and R. Eichenlaub (1980) Plasmid mini-F encoded proteins. Mol. Gen. Genet. 180:205-211.
83. Yarmolinsky, M. (1982) Bacteriophage P1. In Genetic Maps, 2. S.J. O'Brien, ed. National Cancer Institute, National Institutes of Health, Frederick, Maryland, pp. 34-43.
84. Yun, T., and D. Vapnek (1977) Electron microscopic analysis of bacteriophage P1, P1Cm and P7. Determination of genome size,

sequence homology, and location of antibiotic-resistance determinants. Virology 77:376-385.
85. Zyskind, J.W., J.M. Cleary, W.S.A. Brusilow, N.E. Harding, and D.W. Smith (1983) Chromosomal replication origin from the marine bacterium Vibrio harveyi functions in Escherichia coli: oriC consensus sequence. Proc. Natl. Acad. Sci., USA 80:1164-1168.

PARTITIONING OF THE pSC101 PLASMID DURING CELL DIVISION

Stanley N. Cohen,[1] Christine A. Miller,[1] William T. Tucker,[2] Peter A. Meacock,[3] and Peter Gustafsson[4]

[1]Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

INTRODUCTION

It is generally accepted that a mechanism must exist for ensuring that each of the products of a bacterial cell division receives a copy of the chromosome. However, until recently, the existence of a specific mechanism for actively distributing extrachromosomal elements such as plasmids to daughter cells was less clear. Certainly, for plasmids that normally exist in high copy-number within the cell, random distribution alone should be adequate to assure stability in a growing cell population (6,9). However, when the number of individual copies of such plasmids is decreased as a consequence of concatenation (i.e., multimer formation), instability results (1,4,5,12). Addition of a DNA segment that resolves the multimers into monomeric units raises the number of individual copies to a level where random distribution of the copies can accomplish stable inheritance; loci involved in the resolution of multimers, and consequently affecting the extent of plasmid stability, have been identified on the plasmids P1 (1), Clo DF13 (4,5), and ColEI, (12). Similarly, introduction of a strong promoter onto certain high copy-number plasmids is known to interfere with plasmid propagation, decrease copy number, and consequently reduce stability; addition of a terminator downstream from the promoter reverses these effects (13).

---

[2] Research School of Biological Sciences, Australian National University, Canberra City, A.C.T. 2601, Australia; [3] Department of Biochemistry, University of Leicester, Leicester E1 7RH, England; [4] Department of Microbiology, University of Umeå, S-901 87, Umeå, Sweden.

Plasmids having a copy-number too low to accomplish stable inheritance by a random distribution process presumably must employ an active mechanism for this purpose. Loci that encode such a mechanism have been termed par or stb, and have in recent years been identified on the plasmids pSC101 (6), NR1 (7), R1 (9), and F (11). Our investigations of plasmid partitioning have involved pSC101.

Structural Properties of the par Locus of the pSC101 Plasmid

In the initial studies of the par locus of plasmid pSC101, par was assigned to a 370-bp EcoRI/AvaI-generated DNA fragment adjacent to the essential replication region of the plasmid (Fig. 1). Deletion of the DNA segment between the EcoRI site and the proximal HaeII site resulted in plasmid loss from about 75% of cells growing for 100 generations in the absence of selection for the antibiotic-resistance trait encoded by the plasmid (Fig. 2A). Mutants of Par$^-$ pSC101 plasmids that were maintained at a copy number approaching 50 per cell (6) were still found to be unstable, although less so than wild-type pSC101 replicons (Fig. 2B). Thus, even when Par$^-$ pSC101 derivatives were maintained at a copy number that would be expected to fully ensure stable plasmid maintenance by a random distribution model, some instability was nevertheless observed.

The key properties of the pSC101 par locus, as described by Meacock and Cohen (6) are summarized in Tab. 1. The par locus functions normally when placed at sites on pSC101 other than its native location--and it functions equally well when placed in either orientation. However, the presence of the pSC101 par region on a co-existing compatible plasmid does not stabilize a Par$^-$ pSC101 replicon in trans. Furthermore, Par$^-$ high copy-number mutants of pSC101 are unstable despite their elevated copy number, as noted above.

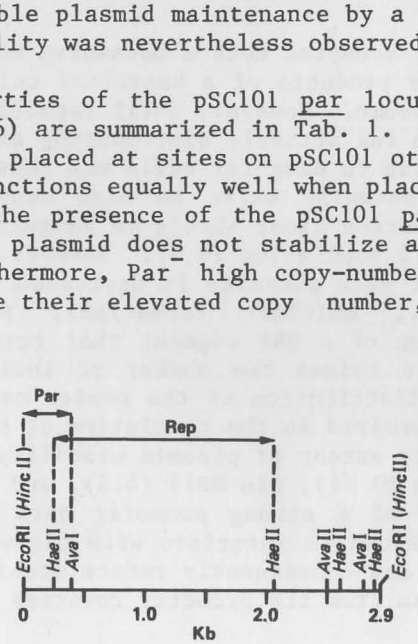


Fig. 1. Map of the HincII-A fragment of pSC101. The location of the cleavage sites of various restriction endonucleases are shown; the HincII sites in pSC101 have been made into EcoRI sites in the pTU1 plasmid. The position of the partition (Par) and replication (Rep) regions are shown. Diagram from Meacock and Cohen (6).

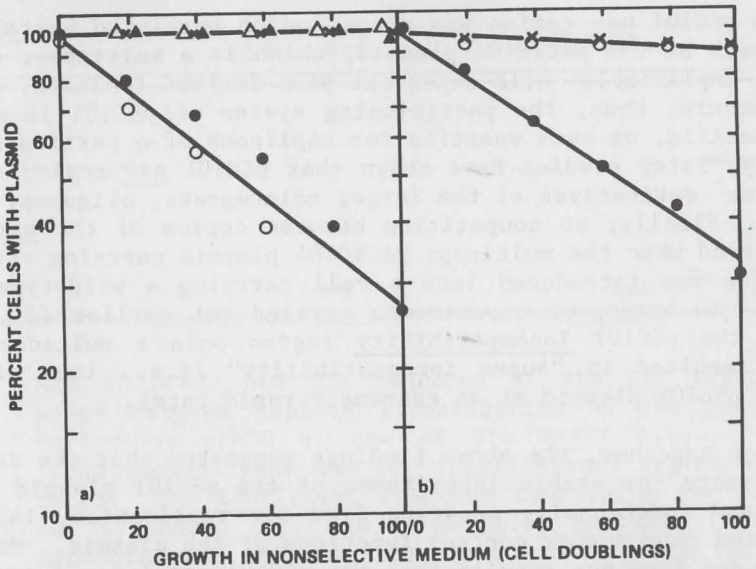


Fig. 2. Stability of autonomously replicating plasmids derived from pSC101 and the effect of an increase in plasmid copy number. Cells of E. coli C600 containing various plasmids were examined for loss of plasmids during growth in non-selective medium by scoring for markers on the plasmids. Various markers on various plasmids were used: Tc^r, pSC101; Km^r, pPM20; Lac$^+$, pPM21; Ap^r, pPM22, pPM23, pPM25, and pPM29. In panel A the rate of loss of plasmids pSC101(×), pPM20(△), pPM21(▲), pPM22(●), and pPM23 (○) is shown. In panel B the rate of loss of plasmid pPM22 (●) is compared to the rates of loss of 2 mutants of pPM22 that have a copy number of 5 times pPM22, pPM25(○), and pPM29(×). Control experiments show that C600 and C600 containing pSC101 show no detectable difference in growth rate. See Meacock and Cohen (6) for experimental details.

Tab. 1. Summary of major properties of PSC101 par locus (6).

1. Works at different locations in cis; non-functional in trans.
2. Works in either orientation.
3. Par$^-$ phenotype not eliminated by elevation in copy number.
4. Stabilizes unrelated plasmids.
5. No instability of pSC101 results from multiple copies of pSC101 par locus on compatible coexisting plasmid.

When the pSC101 par region was placed on an unrelated unstable replicon, such as the pACYC184 plasmid, which is a multicopy, chloramphenicol-amplifiable, polA-dependent p15A-derived replicon, stabilization occurs; thus, the partitioning system of pSC101 is not replicon specific, or even specific for replicons of a particular type. Similarly, later studies have shown that pSC101 par region can stabilize Par$^-$ derivatives of the large, cointegrate, oligocopy plasmid R1 (10). Finally, no competition between copies of the par region was observed when the multicopy pACYC184 plasmid carrying the pSC101 par region was introduced into a cell carrying a wild-type pSC101 plasmid. In analogous experiments carried out earlier (2), placement of the pSC101 incompatibility region onto a multicopy ColE1 plasmid resulted in "super incompatibility" (i.e., loss of a coexisting pSC101 plasmid at an extremely rapid rate).

Taken together, the above findings suggested that the determinant necessary for stable inheritance of the pSC101 plasmid is physically and functionally separate from the replication, incompatibility, and copy number control functions of the plasmid. Moreover, we concluded from our results that the pSC101 par locus does not encode a trans-acting protein; instead, our findings suggested that the locus contains a site that accomplishes even distribution of plasmids to daughter cells in growing bacterial cell populations by interacting with cellularly encoded components of the partitioning system.

DNA Sequence Analysis

DNA sequence analysis has been carried out for the 375-bp EcoRI/AvaI DNA fragment that contains the pSC101 par locus. The resulting sequence shows a number of structurally interesting features. Within the par region are 3 segments that potentially can participate in the formation of hairpin loop structures (8,14). These partition-related (PR) segments have been designated a (location bp 170-185), b (location bp 194-204), and a' (location bp 236-251) in Fig. 3, where the relevant sequences are underscored. The a and a' are direct repeats (14 of 16 bases identical), and b is an inverted repeat of both a and a' (8 of 9 and 9 of 12 bases paired). Thus, hairpin-loop structures can be drawn from both a and b and a' and b. As summarized in Tab. 2, plasmids that have deleted a and a', a and b, or b and a' are phenotypically Par$^-$, but are less unstable than those that have deleted all 3 of the PR segments; the latter deletion mutants show a phenotype that we have termed "Super-par$^-$" (14).

The Super-Par$^-$ Phenotype

Historically, the Par$^-$ phenotype of pSC101 was defined in terms of the plasmid instability displayed by the HaeII replication region fragment shown in Fig. 1. Plasmids containing only this region are

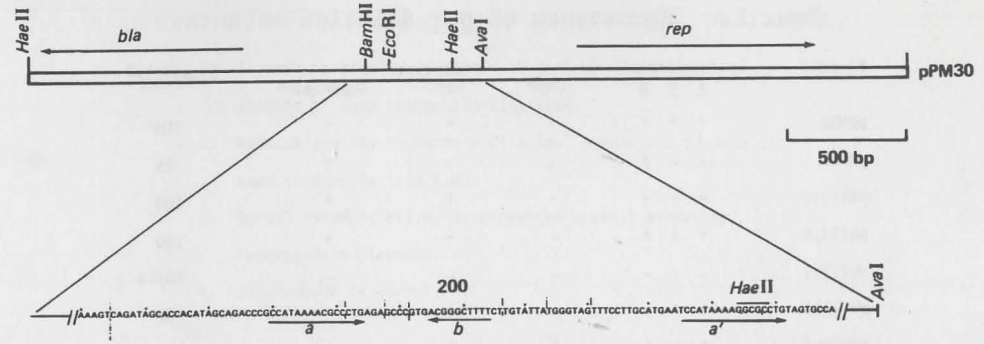


Fig. 3. Map of pPM30 and the sequence of the par region. The upper diagram depicts linearization of the par$^+$ pSC101 derivative pPM30 at one of its HaeII sites. The bla (β-lactamase) gene and rep (replication) region are indicated. The EcoRI/AvaI fragment containing the par locus was sequenced (8), and by deletion analysis (14) the PR segments were defined. They are indicated in this figure by a, b, and a'. Figure modified from Tucker et al. (14).

maintained in 25% of cells grown for 100 generations of the absence of selective pressure (6). However, replicons that lack the entire EcoRI/AvaI par region fragment show a rate of loss that far exceeds that seen with plasmids that lack only the par region segment between the EcoRI (HincII) and HaeII sites. When the entire EcoRI/AvaI fragment is missing, less than 20% of the cells retain the plasmid after 40 generations of growth in nonselective medium, and less than 1% of cells (the limit of detection in the procedure used) contain the plasmid after 60 generations (14). A similar "Super-par$^-$" phenotype was observed when all 3 of the PR segments, a, b, and a', were deleted by BAL31 digestion. Plasmids that have deleted only a or a' are perfectly stable in the absence of a coexisting plasmid derived from the same replicon, and plasmids that have deleted either a and a', a and b, or b and a' display the Par$^-$ phenotype as noted above and previously (14).

The Cmp$^-$ Phenotype

In the course of our studies, we observed that all Par$^-$ plasmids (Gustafsson, Meacock and Cohen, unpub. data), as well as certain pSC101 par deletion mutants having a Par$^+$ phenotype (14), showed a diminished ability to coexist with pSC101 derivatives that contain an intact par region. We have termed this phenotype, which is characterized by an increased sensitivity to incompatibility, Cmp$^-$ for competition; plasmids that are unable to compete on equal terms with another replicon of the same incompatibility group are Cmp$^-$. Cmp$^-$ Par$^+$ plasmids are completely stable for more than 1,000 genera-

Tab. 2. Phenotypes of par deletion mutants.

Plasmid	Sequences[1] a	b	a'	Cmp[2]	Phenotype Par[3]	Super-par[4]	Stability[5]
pPM30	+	+	+	+	+	+	100
pPM22	-	-	p	-	-	+	25
pWTT310	+	+	+	+	+	+	100
pWTT314	+	+	+	+	+	+	100
pWTT315	-	-	-	-	-	-	16/40
pWTT316	+	+	p	-	+	+	100
pWTT317	+	+	+	+	+	+	100
pWTT318	-	-	p	-	-	-	14/40
pWTT319	+	+	p	-	+	+	100
pWTT301	+	+	+	+	+	+	100
pWTT303	-	+	+	-	+	+	100
pWTT304	-	-	+	-	-	+	32
pWTT460	+	+	p	+	+	+	100
pWTT461	+	+	p	-	+	+	100
pWTT462	+	+	-	-	+	+	100
pWTT463	+	+	-	-	+	+	100
pWTT464	+	-	-	-	-	+	29
pWTT465	p	-	-	-	-	+	10
pWTT466	-	-	-	-	-	-	20/40
pJGB11	-	+	p	-	-	+	79
pJGB12	-	+	+	-	+	+	100
pJGB111	-	+	p	-	-	+	15

[1]The sequence of the par region is shown in Fig. 3. In it the 3 PR (partition related) sequences are underlined and marked a, b, and a'. To show how much of these sequences is present in various plasmids, a "+" indicates the sequence is present, a "-" indicates it is deleted, and a "p" indicates it is partially present.

[2]Cmp phenotype is measured by a plasmid's ability to transform the strain PM191 carrying the incompatible wild-type pSC101 plasmid compared to its ability to transform the host strain PM191. Normal incompatibility reduces this number by approximately 10-fold; in cases where the transforming ability of a plasmid is reduced more than 1,000-fold, that plasmid is called Cmp^-.

[3]Par phenotype is an indication of the plasmid's stability over 100 generations of unselected growth.

Tab. 3. Characteristics of the Cmp^- phenotype.

1. Plasmid is totally stable in absence of coexisting Cmp^+ plasmid of same incompatibility type.
2. Reduces ability to compete with Cmp^+ coexisting plasmid of same incompatibility type.
3. Normal incompatibility is expressed against other Cmp^- incompatible plasmids.
4. Copy number is normal.

tions of growth in nonselective media. However, they show a reduction of transformation frequency that exceeds by 3 orders of magnitude the reduction normally observed when a wild-type plasmid is introduced into cells already harboring an incompatible wild-type plasmid. The characteristics of the Cmp^- phenotype are summarized in Tab. 3.

The Cmp phenotype of pSC101 is determined by sequences within its par region and appears to represent an intermediate stage of expression of the par function (14). When only one PR segment is deleted, a Cmp^- plasmid is formed; however, when 2 of the PR segments

[4]The $Super\text{-}par^-$ phenotype reflects a degree of instability so great that after 100 generations, less than 1% of the cells growing in the absence of selection contain the plasmid.

[5]Stability is the percent of cells retaining the plasmid, measured by percent of Ap^r cells after 100 generations of unselected growth; each is an average of 3 separate experiments in which less than 10% deviation was observed. When the Par value would be 0 in 100 generations, we took the last time point that yielded any ampicillin-resistant cells and expressed the value in Ap^r cells in that number of generations of unselected growth (e.g., 16/40 is 16% Ap^r cells after 40 generations).

are deleted, a Par$^-$ plasmid results. However, the Cmp phenotype also appears to be associated in some way with the replication/incompatibility system of the plasmid, since a Cmp$^-$ pSC101 derivative coexists stably with pACYC184-derived replicon that carries the *par* region of pSC101.

Apparent Plasmid Pool Size vs. Actual Pool Size in par$^-$ Mutants

As indicated in Fig. 2 and Tab. 1, when a *par*$^-$ derivative of pSC101 that is maintained at 5 times the number of copies as the wild type was examined, some instability was observed--despite the calculation that random distribution of the multiple copies of the plasmid should not have resulted in its loss from any of the cells in the population (6). Super-par$^-$ derivatives of pSC101 that carry a wild-type replication region are lost at a rate consistent with random distribution of a replicon having a copy number of 4 per cell at division, whereas the actual copy number of the plasmid was measured at several times this value (14). Thus, the number of copies in the intracellular pool of plasmids does not seem to be taken into account in the segregation of Super-par$^-$ mutants. Similarly, direct examination of the effects of copy number on the stability of Par$^-$ and Super-par$^-$ mutants confirmed that high copy-number mutations have only a small effect on the rate of loss of a Super-par$^-$ mutant, supporting the view that the separate plasmids in the intracellular pool are not individually "counted" in the absence of *par*; an increase in plasmids from 15 copies per cell at division to 5 times this number of copies does not yield a correspondingly slower rate of loss (14).

Studies with a Super-par$^-$ derivative of pSC101 that also carries a temperature-sensitive mutation in the replication region support this interpretation; following abrupt inhibition of plasmid replication by shift to the nonpermissive temperature, half of the cell population loses the Super-par$^-$ *ts* plasmid in the first generation following the temperature shift (Fig. 4). In contrast, a *par*$^+$ *ts* derivative of pSC101 was lost from cells growing at the nonpermissive temperature at a rate consistent with a plasmid copy number averaging about 10 per cell; for more than 2 doublings after the temperature shift, most cells retained a copy of this plasmid. During each subsequent generation, the fraction of cells retaining the plasmid decreased by half.

It has already been noted that the loss of ColE1, pACYC184, and other high copy-number plasmids from growing cell populations is often associated with the formation of multimers; such multimerization apparently reduces the number of separate plasmid molecules to a level where random distribution can no longer ensure stable inheritance of these plasmids. Introduction of a plasmid locus that accomplishes resolution of the multimers into unit-length molecules restores stability (4,5,12). However, as shown in Fig. 5, stabilization of the pACYC184 plasmid by the pSC101 *par* region does not occur by resolution of pACYC184 multimers to monomeric molecules. Thus, plasmid stabilization by the pSC101 *par* region does not appear to involve a mechanism that regulates the number of individual plasmid molecules in the cell. This conclusion is consistent with our failure to observe multimers of pSC101 derivatives that lack the *par* region (14).

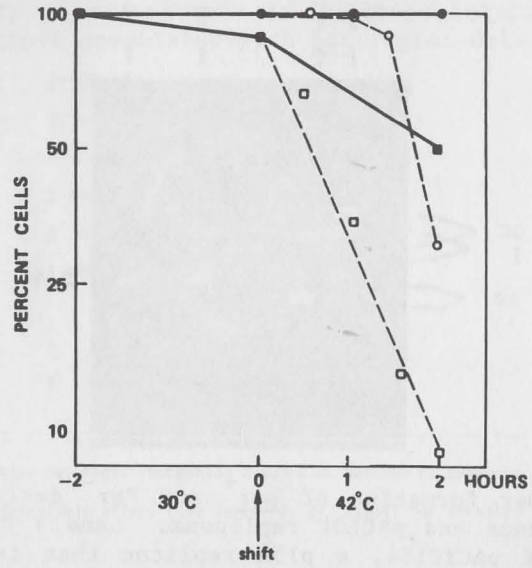


Fig. 4. Rate of loss of Par$^+$ vs. Super-par$^-$ *ts* pSC101-derivative plasmids upon shift to the nonpermissive temperature. PM191 (a *recA*$^-$ derivative of C600) carrying plasmids pCM301 (Par$^+$) or pCM302 (Super-par$^-$) which are *ts* for replication, were grown at 30°C without antibiotics. After 2 hours, half of each culture was shifted to 42°C. Samples were taken at the time points shown, plated on nonselective media, and the resulting colonies were tested for resistance to Ap to determine the presence of the plasmid. Circles are pCM301, squares are pCM302. Solid lines and closed symbols represent growth at 30°C; dotted lines and open symbols represent growth at 42°C. The generation time at 42°C was approximately 30 min. Figure from Tucker et al. (14).

DISCUSSION

It is now apparent that at least 3 discrete segments of DNA within the *par* region of pSC101 are involved in partitioning of the

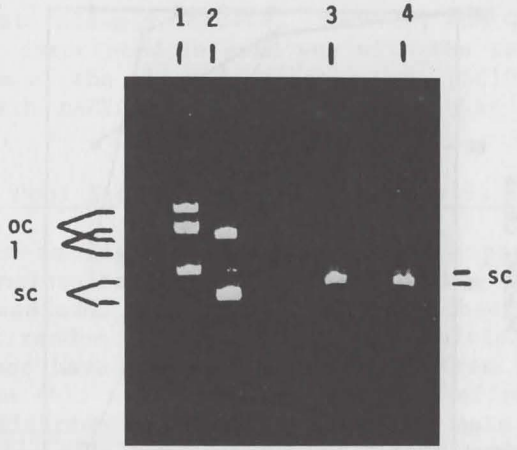


Fig. 5. Multimer formation of par$^+$ and Par$^-$ derivatives of p15A replicons and pSC101 replicons. Lane 1 contains plasmid DNA of pACYC184, a p15A replicon that is unstable, and Lane 2 contains plasmid DNA of pPM31, a derivative of pACYC184 that has the pSC101 par region and is stable. The prominent bands are the supercoiled, open circular, and linear DNA, as indicated, while the less prominent bands indicate various multimers. Details about these plasmids are reported in Chang and Cohen (3) and Meacock and Cohen (6). Lane 3 contains plasmid DNA of pPM30, a par$^+$ pSC101 replicon, and Lane 4 contains plasmid DNA of pWTT315, a Super-par$^-$ pSC101 replicon. The prominent band is the supercoiled DNA and no multimers of either plasmid are detectable. These plasmids are described in Meacock and Cohen (6) and Tucker et al. (14).

plasmid to daughter cells. Deletion of all 3 segments results in the Super-par$^-$ phenotype, which is also observed with plasmids that lack the entire EcoRI/AvaI segment. Deletion of 2 of the PR segments yields the simple Par$^-$ phenotype. Plasmids that have only one of the PR segments deleted are perfectly stable when in the absence of coexisting wild-type plasmid derived from the same replicon, but show a decreased ability to compete with incompatible coexisting plasmids (the Cmp phenotype). The structural and functional interrelationships of the phenotypes associated with par region deletions are summarized in Tab. 4.

Our results suggest a model in which the par function of the pSC101 plasmid enables the nonreplicating plasmids of the intracellular pool to be: 1) counted as individual molecules, and 2) distributed evenly to daughter cells. Moreover, our data with par$^-$ ts plasmids suggest that the plasmid being replicated at the time of

Tab. 4. Summary of structural and function interrelationships of phenotypes associated with par region deletions.

Deletion*	Phenotype
a, b, and a'	Super-par$^-$
a and b	Par$^-$
a' and b	Par$^-$
a and a'	Par$^-$
a	Cmp$^-$
a'	Cmp$^-$

*Complete deletion of PR segment yields greater effects than partial deletion. Extending a deletion between PR segments has no detectable effect (see Tucker et al. (1984) for details).

cell division may be distributed separately from the pool and randomly with regard to the pool.

We view the Super-par$^-$ phenotype as the result of total ablation of par function, so that plasmids having this phenotype behave entirely and quantitatively according to the proposed model. The simple Par$^-$ phenotype represents a lesser degree of loss of function; there is some capacity for counting and partitioning the individual plasmids in the nonreplicating pool Presumably, the presence of a single intact PR segment in simple Par$^-$ mutants is sufficient to impart some degree of par function.

While the above model is speculative, it is consistent with our observations. However, the applicability of the model to the Cmp$^-$ phenotype is less direct. While previously it has been shown that the par region of the pSC101 plasmid is able to stabilize Par$^-$ plasmids that utilize an entirely different mode of replication, the discovery of the Cmp$^-$ phenotype suggests that the partition and the replication/incompatibility mechanisms for the pSC101 plasmid nevertheless have some degree of functional linkage. When a Cmp$^-$ plasmid is present in the same cell as a wild-type derivative of the same replicon, the Cmp$^-$ plasmid is at a competitive disadvantage. However, when 2 replicatively similar Cmp$^-$ plasmids coexist in the same cell, they behave identically to 2 coexisting Cmp$^+$ plasmids and show normal incompatibility properties (14). The exact nature of the replicative disadvantage of the Cmp$^-$ plasmid remains to be elucidated, as does the mechanism by which the PR segments a, b, and a' accomplish the partitioning of the pSC101 plasmid during the active cell growth.

ACKNOWLEDGMENTS

These studies were supported by Grant GM26355 to S.N.C. During part of this study, P.A.M. was the recipient of a NATO Fellowship from the Science Research Council of the United Kingdom, and P.G. was the recipient of a Postdoctoral Fellowship Award from the Medical Research Council of Denmark.

REFERENCES

1. Austin, S., M. Ziese, and N. Sternberg (1981) A novel role for site-specific recombination in maintenance of bacterial replicons. Cell 25:729-736.
2. Cabello, F., K. Timmis, and S.N. Cohen (1976) Replication control in a composite plasmid constructed by in vitro linkage of 2 distinct replicons. Nature 259:285-290.
3. Chang, A.C.Y., and S.N. Cohen (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bact. 134:1141-1156.
4. Hakkaart, M.J.J., P.J.M. vanden Elzen, E. Veltkamp, and H.J.J. Nijkamp (1984) Maintenance of the bacteriocinogenic plasmid Clo DF13 in E. coli cells: Evidence for Site-Specific Recombination at parB. Cell 36:203-209.
5. Hakkaart, M.J.J., E. Veltkamp, and H.J.J. Nijkamp (1982) Maintenance of the bacteriocinogenic plasmid Clo DF13 in Escherichia coli cells. II. Specific recombination functions involved in plasmid maintenance. Mol. Gen. Genet. 188:338-344.
6. Meacock, P.A., and S.N. Cohen (1980) Partitioning of bacterial plasmids during cell division. A cis-acting locus that accomplishes stable plasmid inheritance in cell populations. Cell 20:529-542.
7. Miki, T., A.M. Easton, and R.H. Rownd (1980) Cloning of replication, incompatibility and stability functions of R plasmid NR1. J. Bact. 141:87-99.
8. Miller, C.A., W.T. Tucker, P.A. Meacock, P. Gustafsson, and S.N. Cohen (1983) Nucleotide sequence of the partition locus of Escherichia coli plasmid pSC101. Gene 24:309-315.
9. Nordström, K., S. Molin, and H. Aagard-Hansen (1980) Partitioning of plasmid R1 in Escherichia coli: I. Kinetics of loss of plasmid derivatives deleted of the par region. Plasmid 4:215-227.
10. Nordström, K., S. Molin, and H. Aagard-Hansen (1981) Plasmid R1 incompatibility. Contribution from the cop/rep and from the par systems. In Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, R.C. Clowes, S.B. Levy, and E.L. Koenig eds. Plenum, New York, pp. 291-301.
11. Ogura, T., and S. Hiraga (1983) Partition mechanism of F plasmid: Two plasmid gene-encoded products and a cis-acting region are involved in partition. Cell 32:351-360.

12. Summers, D.K., and D.J. Sherratt (1984) Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability. Cell 36:1097-1103.
13. Stueber, D., and H. Bujard (1982) Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. EMBO Vol. 1, No. 11, pp. 1399-1404.
14. Tucker, W.T., C.A. Miller, and S.N. Cohen (1984) Structural and functional analysis of the par region of the pSC101 plasmid. Cell 38:191-201.

DNA-PROTEIN INTERACTION AT THE REPLICATION ORIGINS OF PLASMID CHROMOSOMES

D. Bastia, C. Vocke, J. Germino, and J. Gray

Department of Microbiology and Immunology
Duke University Medical Center
Durham, North Carolina 27710

ABSTRACT

Novel techniques have been developed to purify replication initiator proteins of the plasmids R6K and pSC101. The techniques consist of tagging the initiator cistrons at the C-terminus with β-galactosidase-encoding DNA of Escherichia coli in the correct translational phase. The hybrid proteins are then rapidly purified by adsorption to and elution from a β-galactosidase- specific affinity column. Two procedures have been devised to isolate the nonfused initiator proteins using the fused protein as a handle. The first procedure, called subunit association chromatography, exploits the association of a monomer of nontagged protein with that of β-galactosidase-tagged protein in isolating both types of proteins by β-galactosidase specific affinity column chromatography. The second procedure involves the fusion of the initiator protein to β-galactosidase via a specific linker DNA. The linker DNA encodes a protein which is readily and specifically hydrolyzed by a sequence specific protease, thus releasing the initiator protein from β-galactosidase. Using purified or partially purified initiator protein, we have demonstrated that the R6K encoded initiator protein (Pi protein) binds to a consensus 22 bp sequence at 2 regions of the plasmid chromosome. The pSC101-encoded initiator protein binds to sequences at or near the plasmid replication origin. At low concentrations the protein binds to a nucleation site and upon raising the concentrations of the protein binding is promoted at 4 adjacent sequences that have partial homologies with the nucleation sequence. Deletion of the binding site leads to a nonfunctional replication origin.

INTRODUCTION

The mechanism of initiation of Cairns-type DNA replication continues to be an interesting unsolved problem in molecular biology. In order to study this problem we have concentrated on the purification of initiator proteins from 2 plasmid systems namely R6K (1) and pSC101 (2,3).

The plasmid R6K is 26 x 10^6 daltons in molecular weight and contains 3 replication origins (4,5,6), a unique replication terminus (7,8,9,10), and encodes a 35-kd initiator protein also known as Pi protein (11,12,13).

The plasmid pSC101 discovered by Cohen and co-worker (2,3) is a low copy-number replicon that is dependent on the E. coli dnaA protein (14) and a plasmid-coded initiator protein (15,16) for its replication. The primary structure of the plasmid-coded initiator has been determined from its nucleotide sequence and the protein has been partially purified (15). The minimal replicon of pSC101 was previously localized in a 1800-bp autonomously replicating fragment (17). The topology of this fragment has been determined both structurally and functionally by nucleotide sequence analysis and in vitro deletion analysis (15).

In this chapter, we present novel procedures for the purification of plasmid-encoded initiator proteins and summarize results pertaining to the interaction of these proteins with their respective binding sites on the plasmid chromosomes.

RESULTS

Purification of the R6K Initiator Protein (Pi Protein) by Subunit Association Chromatography

The principle of this purification procedure is to express 2 copies of the R6K initiator cistron, one of which is fused to the lac Z cistron, in tandem under the transcriptional control of the lac promoter. Since the R6K initiator is polymeric in structure we expected that the nonfused initiator protein would associate by subunit interaction with the β-galactosidase-fused initiator subunit. Because of this association, both types of protein should be recoverable in a relatively pure form by β-galactosidase specific affinity column chromatography. With this approach in mind, we constructed the plasmid pJG11 which contains 2 tandem copies of the R6K initiator cistron, one of which is fused to β-galactosidase (18) in the correct translational phase (Fig. 1). Both the fused and nonfused cistrons are under the transcriptional control of the lac promoter. The analysis of the proteins isolated from cells that carry the plasmid pJG11 shows that affinity chromatography in pNH_2-Phe-S-gal. (p-amino-phenyl-thio-galactoside-sepharose) column indeed purifies both the β-galactosidase tagged and the nontagged initiator proteins (Fig. 1). Amino-terminal amino acid sequence analysis confirms that the 35-kd molecular weight protein has the same first 10 amino acids as the β-galactosidase tagged 160-kd protein (18).

Purification of the R6K Initiator by Gene Fusion and Site-Specific Proteolysis

We wished to develop a more general procedure for the rapid purification of proteins from recombinant DNA clones. Our approach was to fuse the R6K initiator cistron to the β-galactosidase cistron via a piece of DNA encoding the pro α-2 collagen of chicken. The tripartite fusion cistron was in the correct translational phase and under the transcriptional control of the λP_R promoter in the plasmid pJG201 (Fig. 2).

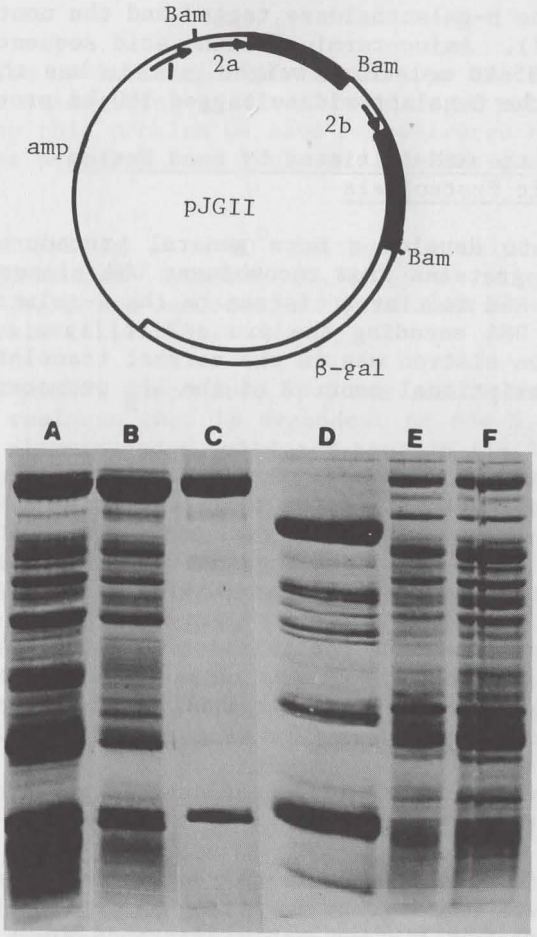
The λP_R promoter was activated by raising the temperature to 42°C and thereby inactivating the thermosensitive λ CI857 repressor. The tripartite fusion protein was purified by β-galactosidase-specific affinity column chromatography and then cleaved with collagenase of Achromobactor iophagus. Microbial collagenase are known to cleave specifically the sequence pro-X-gly-pro-Y (19). The collagenase cleavage separated the initiator protein moiety from β-galactosidase. Column chromatography on a TSK 4000 column using high performance liquid chromatography (HPLC) enabled us to purify the R6K initiator in a homogenous form (Fig. 3 and Ref. 20).

Biological Activity of the β-Galactosidase-Tagged R6K-Encoded Initiator Protein

The biological activity of the β-galactosidase-tagged initiator protein was examined by in vitro DNA replication assay and by sequence-specific DNA binding assay described in a subsequent section. In vitro replication of R6K DNA was previously demonstrated in crude cell extracts (21). We wished to determine if the β-galactosidase-tagged initiator protein of R6K was competent to initiate DNA replication. The conditions of the in vitro replication reaction are shown in the legend to Fig. 4. The results show that the β-galactosidase-tagged initiator protein promotes rifampicin-sensitive and novobiocin-sensitive DNA synthesis. We have analyzed the reaction products by 2-dimensional neutral-neutral and neutral-alkaline agarose gel electrophoresis and confirmed that a full range of replication intermediates are formed and these appear to be Cairns-type intermediates (18).

Sequence-Specific DNA-Binding Activity of the R6K Initiator Protein and Location of the DNA-Binding Domain

We have previously demonstrated that the β-galactosidase-tagged initiator protein of R6K binds to 2 specific regions located at the 5' untranslated region of the initiator cistron (19,22). In this



chapter, we summarize the DNA-protein interaction studies performed with both the β-galactosidase-tagged protein and the collagenase - cleaved purified initiator protein. We further demonstrate that the DNA binding domain of the R6K initiator is located in the C-terminal 2/3rd of the protein. The amino terminal region of the initiator protein, unlike the lac and λ repressor proteins (23,24), did not show consistent sequence-specific DNA-binding.

In order to compare the DNA-binding characteristics of the β-galactosidase-tagged protein and the nontagged purified initiator we

Fig. 1. Upper - Structure of the plasmid pJG11. The plasmid contains 2 copies of the initiator protein cistron of R6K in the correct transcriptional phase with the lac promoter [1]. The second copy of the initiator cistron is fused to the lac Z cistron in the correct translational phase at the 9th codon from the N terminus of the lac Z gene. Each initiator cistron also contains its own natural promoter [2a, 2b].

Lower, SDS-polyacrylamide gel analysis of proteins produced by E. coli (lac Z) cells carrying the plasmid pJG11. A, total protein; B, a 40% $(NH_4)_2SO_4$ precipitate; C, protein purified by affinity column chromatography through a pNH_2PheS gal column (Ref. 18). Note the simultaneous isolation of the 160-kd β-galactosidase tagged protein and the 35-kd R6K initiator which is not tagged with β-galactosidase, presumably because of subunit association between the 2 types of protein monomers.

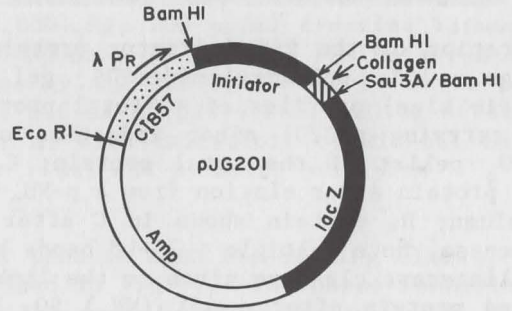


Fig. 2. Structure of the plasmid pJG201. The plasmid contains the initiator cistron of R6K fused at the Bam H1 site in the correct translational phase with the linker DNA sequence coding for 60 amino acids from the triple helical region of chicken pro-α-2 collagen. The linker has several units of sequence of the type pro-X-gly-pro-y, which is specifically cleaved by microbial collagenase. The collagen linker coding sequence is fused to the lac Z gene at the 9th codon from the N-terminus and in the correct translational phase.

Thus pJG201 encodes a tripartite protein under the transcriptional control of the inducible λ P_R promoter. The plasmid carries the thermosensitive λ CI857 repressor gene, the ribosome binding site of Cro and the ATG of Cro which is not in phase with the tripartite hybrid protein.

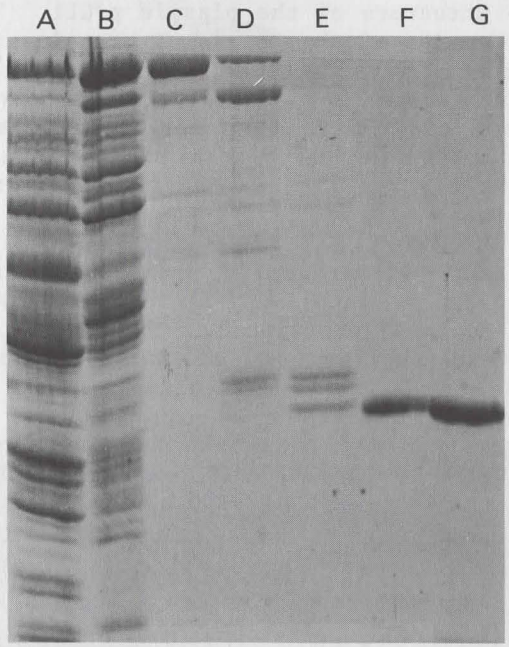


Fig. 3. Purification of the R6K initiator protein by collagenase cleavage. 10% polyadrylamide-SDS gel (stained with Coomassie blue) profiles of A, total protein from E. coli cells carrying pJG201 after induction of λ P_R; B, 40% $(NH_4)SO_4$ pellet of the total protein; C, the tripartite hybrid protein after elution from a p-NH_2-Phe-S-gal affinity column; D, protein shown in C after cleaverage with collagenase, note multiple ∿ 35-kd bands because of multiple collagenase cleavage sites in the linker; E, partially purified protein after a 40% (NH_4) SO_4 precipitation of the protein shown in E, the protein in the supernatant is shown; F, G, 15 µg and 30 µg of purified R6K initiator after fractionating the protein shown in B through a TSK 3000 HPLC gel filtration column.

performed nitrocellulose filter binding experiments (22,25). The results of these experiments depicted in Fig. 5 show that both the β-galactosidase-tagged and nontagged initiator bind to identical restriction fragments belonging to the region called Site I and Site II (22,26). Therefore, as previously noted by us (26), the presence of the β-galactosidase has no detectable influence on the sequence-specific DNA binding of the R6K initiator protein.

We have performed footprinting experiments (Fig. 6) and methylation protection experiments to further analyze the interaction of

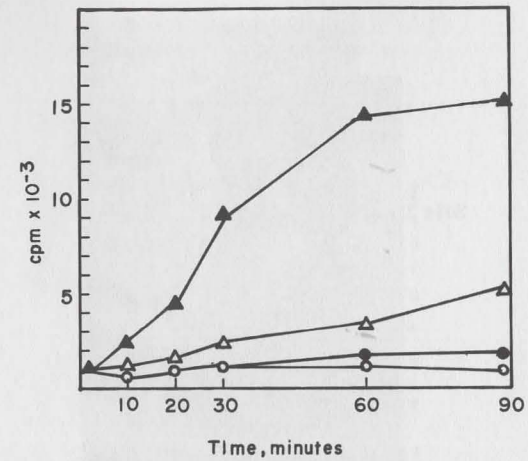


Fig. 4. In vitro replication of the plasmid pJG14 (ori γ cloned in pUC9). Cell lysates were prepared by the lysozyme-freeze-thaw method from the strain D110 (E. coli Pol A^-) as described before (18). The 50 µl reaction mixture contained [α-^{32}P] dATP; 200 µm each of dCTP, TTP, and dGTP; 500 µm each of GTP, CTP, UTP; 2 mM ATP; 40 mM Hepes (pH 7.6); 11 mM $(CH_3COO)_2Mg$; 100 µg/ml creatine kinase; 21.6 mM creatine phosphate; 50 µg/ml of bovine serum albumin; 6% polyethylene gylcol 8,000; 200 µg of crude protein from cell lysate; 8.6 µg of pJG14 DNA; 420 µg of initiator (β-gal.-tagged; ▲, with initiator; △, without initiator; ○, rifampicin plus; and ●, navobiocin plus.

the R6K-initiator protein with the binding Sites I and II. The results are summarized in Fig. 7. The DNase footprinting experiments demonstrate that the protein interacts with the consensus sequence APuNCATGAGPyTTAGTNCGTN. The binding Site I contains 7 tandem copies of this sequence (27) while the Site II contains one complete copy and 2 partial copies of these sequences (Fig. 7; Ref. 22). Interaction with the initiator protein makes the 10th residue from the left of the top strand and 3rd residue from the right of the bottom strand of each 22 bp repeat specifically "hypersensitive" to digestion by DNase I thus suggesting some structural alterations such as bending or kinking of the binding site.

Methylation protection studies show that the protein specifically makes contact with purine residues located mostly on the top strand of the DNA (Fig. 7; Ref. 22).

In order to localize the DNA-binding domain of the protein, we digested the initiator cistron at the single Bgl II site (Fig. 8) and separately cloned the N-terminal and C-terminal pieces in the

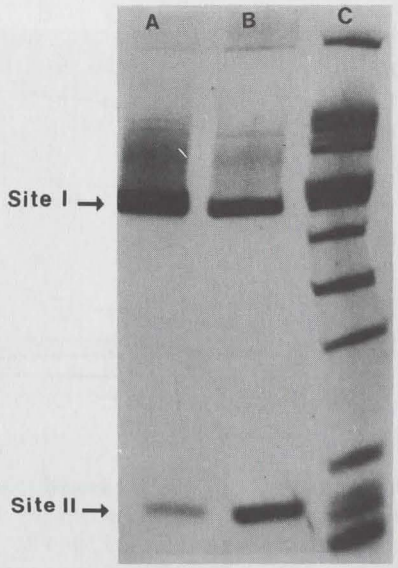


Fig. 5. Interaction of R6K initiator protein with R6K DNA. Autoradiogram of a 5% polyacrylamide gel of 3' end labeled, Hind III-Hinf I restriction fragments of the plasmid pJG4 (which contains the β-replicon of R6K cloned in pBR322, see Ref. 26). A, DNA fragments bound to purified R6K initiator, after collagenase cleavage and retained on a nitrocellulose filter; B, DNA fragments bound by the tripartite hybrid protein (Fig. 3C); C, Input 3'-end labeled restriction fragments. Thus both the β-galactosidase tagged initiator and purified nontagged initiator both bind to identical restriction fragments (Site I and Site II).

vector pJG200 (20) in the correct translational phases with the collagen and β-galactosidase sequences. Both types of the protein were purified using the procedures described before (20) and were used to perform nitrocellulose filter-binding experiments. The results depicted in Fig. 9 demonstrates that the C-terminal fragment, but not the N-terminal fragment, shows sequence specific DNA binding. Thus, the R6K-initiator (Pi protein) contains at least one sequence-specific DNA-binding domain in the two-thirds of the peptide from the carboxy-terminus.

The Replication Origin and Initiation Protein of Plasmid pSC101

The replication origin of the plasmid pSC101 was previously localized in a 1,800-bp autonomously replicating fragment by Cohen and co-workers (17). This fragment lacked the partition locus

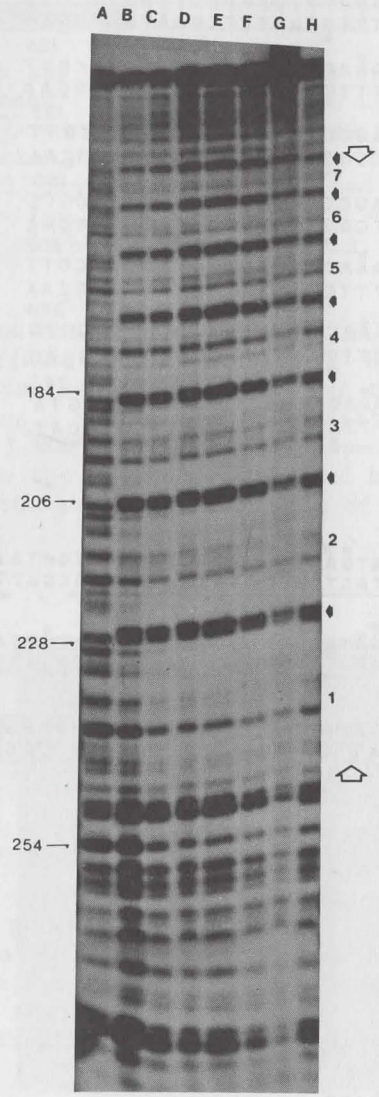


Fig. 6. DNase I footprint of the β-galactosidase tagged R6K initiator at binding Site I. The restriction fragment containing Site I was 5'-end labeled and digested with DNase I (panel A) or was first complexed with increasing amounts of initiator protein and then cleaved with DNase I (B-H). Note, 7 tandem protected regions in B-H each of which are bounded by a residue that is hypersensitive to DNase I (→). See Fig. 7 for more details. The numbers on the left correspond to the coordinates shown in Fig. 7.

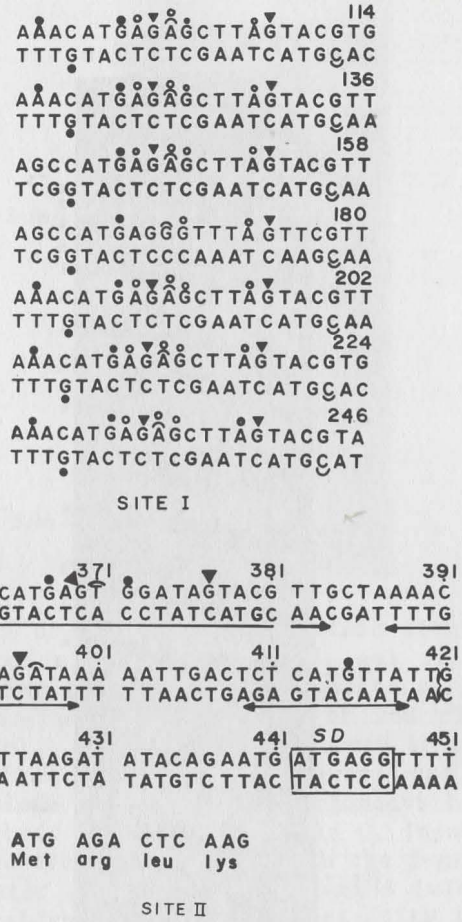


Fig. 7. Binding Sites I and II of R6K showing the summary of footprinting and methylation protection experiments. υ, η = enhanced sensitivity to DNase I, ● = strongly blocked from methylation; ○ = weakly blocked from methylation; ▼ = enhanced methylation; SD = Shine-Delgarno sequence. The region protected from DNase digestion at Site II is shown within brackets.

(17,28). We have further dissected the replication origin by nucleotide sequence analysis and in vitro mutagenesis. The functional replicon of pSC101 consists of a trans-acting initiator cistron (Fig. 10; and Ref. 16) and a cis-acting origin (Fig. 10; Ref. 15, 16). The origin region consists of an AT-rich region, a putative dnaA binding site and the binding sites for the pSC101-encoded initiator protein (Fig. 10).

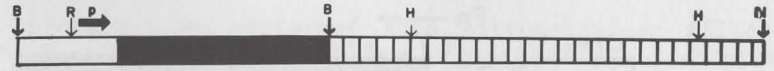


Fig. 8. Restriction map of the R6K initiator cistron. The N-terminal fragment (▬) and the C-terminal DNA fragment (▭) were separately cloned into the vector pJG200 which contains the collagen linker (Ref. 20). The N- and C- terminal peptides were purified as described in the text. R_1 = Eco R_I, Bl = Bam HI, B = Bgl I, S = Sau 3A, p = promoter of the initiator cistron.

In vitro deletion analyses showed that all sequences beyond the cistron for the initiator are dispensable. However, deletions extending into the coding region corresponding to the C-terminus give nonfunctional replicon. Similarly, up to approximately 100 bp from the left end of the 1,800-bp region are dispensable. However, when deletions extend into the AT-rich region and beyond of the untranslated sequences, inactivation of the origin of replication (Fig. 10) takes place.

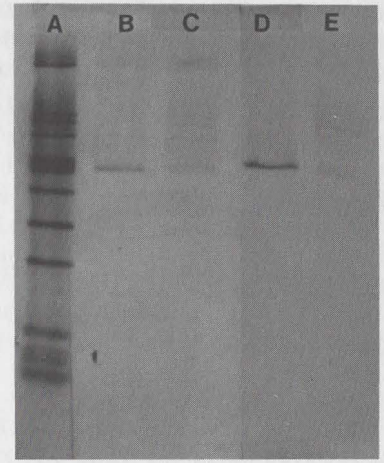


Fig. 9. Interaction of the C-terminal and N-terminal peptides of R6K initiator (see Fig. 8) with DNA. Autoradiograms of a 5% polyacrylamide gel showing A, input DNA; B, D, restriction fragment bound to C-terminal peptide; and C, E, restriction fragment bound to the N-terminal peptide. Note that only the C-terminal peptide shows sequence-specific DNA-binding.

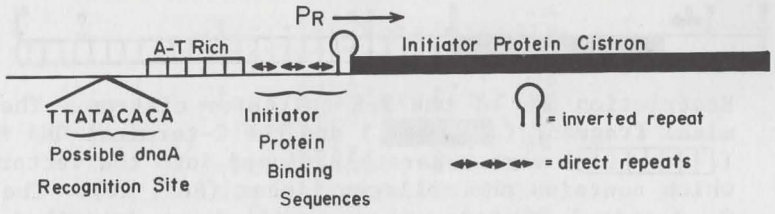


Fig. 10. The replication origin region of the plasmid pSC101. Note the AT-rich region, putative dnaA binding sites, the plasmid-encoded protein binding site, and the 3 direct repeats (→).

The Initiator Protein of pSC101

Using the techniques mentioned earlier, we have partially purified the replication initiator (37.5 kd) protein of pSC101 and studied its interaction with the replication origin.

We constructed the plasmid pCV2 (Fig. 11; Ref. 29) which contains the 1,800-bp autonomously replicating fragment of pSC101 cloned into the vector pUC9 (30). The HinfI-generated restriction sites of pCV2 are shown (Fig. 11). The pCV2 DNA was digested with HinfI and 3'-end labeled with [α-p^{32}] dATP and the labeled DNA was used to study DNA-protein interactions with the pSC101-encoded initiator protein. We performed both nitrocellulose filter-binding and anti-β-galactosidase antiserum promoted immunoprecipitation of β-galactosidase-tagged initiator DNA complex to localize the binding region on the pSC101 DNA. The results showed that the Hinf 1B fragment specifically binds to the plasmid-encoded initiator protein (29).

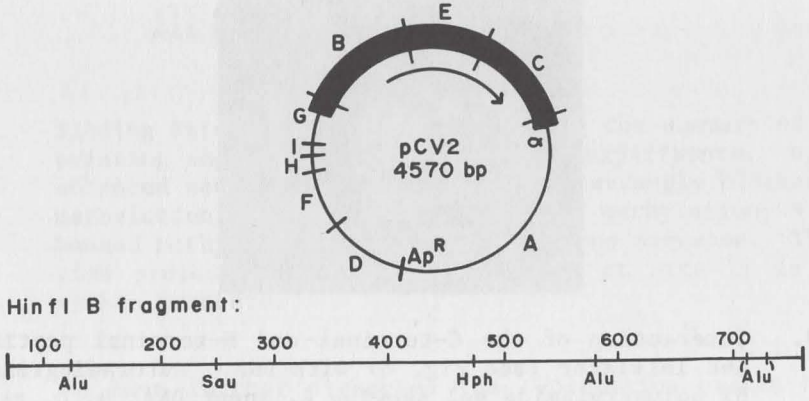


Fig. 11 Restriction (HinfI) map of the plasmid pCV2. pSC101 replicon (■), pUC9 vector DNA (□). Alu = Alu I, Sau = Sau 3A, Hph = HphI, Ap^R = β-lactamase gene, α = α-donor (peptide) gene of β-galactosidase. The Hinf 1B fragment specifically binds to the pSC101-encoded 37.5-kd initiator protein (Ref. 29).

In order to study the interaction of the protein with DNA as a function of the input protein concentrations, we performed exonuclease III-footprinting as described in the legends to Fig. 12. The results show that at the lowest protein concentration, the protein occupies a nucleation site. Upon increasing the protein concentration the protein binds to 4 adjacent sites.

We also performed methylation protection and interference experiments (29) to delineate the pattern of interaction of the protein with DNA. The results are summarized in Fig. 13.

The first 3 direct repeats are homologous to each other but they share partial homologies with the nucleation site and the intervening binding sites.

DISCUSSION

The protein purification procedure described in this chapter does not depend on the biological activity of the protein being purified but is based on the assay and specific affinity procedure for β-galactosidase which is used as a tag. Therefore, the procedures should be applicable to other types of proteins. Since β-galactosidase of *E. coli* is expressed in yeast cells, in mammalian cells, and in Drosophila (31, 32, 33), with appropriate modifications of the vector (selectable marker and proper replication origins), the techniques could be used to purify proteins from these eukaryotic cells.

The apparent limitation of the subunit association chromatography is that it would only be effective if the target protein is polymeric in structure. This limitation has been overcome to a large extent by the use of the second approach that uses the collagen linker. Previous work, using Clostridial collagenase and synthetic peptides of defined sequences, has shown that collagenase fractions were found that selectively hydrolyzed the sequence pro-X-gly-pro-y (19). Very few proteins other than collagen have these sequences (34). Therefore, the collagenase-linker technique should be useful for the purification of a wide range of proteins provided that the addition of the pro-X to the C-terminus, resulting from collagenase cleavage did not impair the biological activity of the target protein.

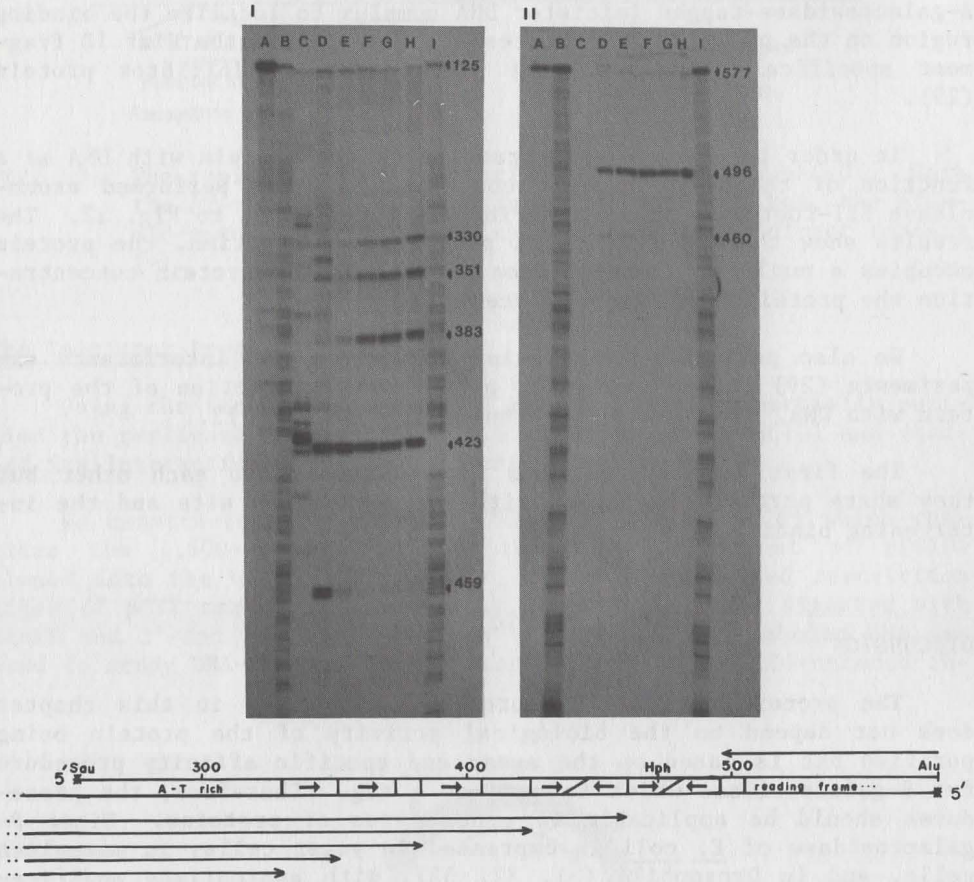


Fig. 12. Exonuclease III footprint of the pSC101-encoded initiator protein at the replication origin of pSC101 revealing 5 binding sites and possible cooperative binding. Autoradiograms of 8% Acrylamide - 7M Urea gels showing, (I) pSC101 origin DNA 5'-end labeled at the Alu I end (see panel III), (II) DNA 5'-end labeled at the Sau 3A site (see III), (III) The ori region that is end labeled A - Undigested DNA; B and I, G>A Maxam-Gilbert cleavage reaction supplying molecular weight markers; C - exo-III digestion of DNA without added initiator; D-H, exo-III digestion in the presence of increasing amounts of initiator protein. Note that in panel I the band (corresponding to coordinate 459) protected at the lowest protein concentration is the nucleation site. Note, 4 additional sites protected with increasing protein concentrations.

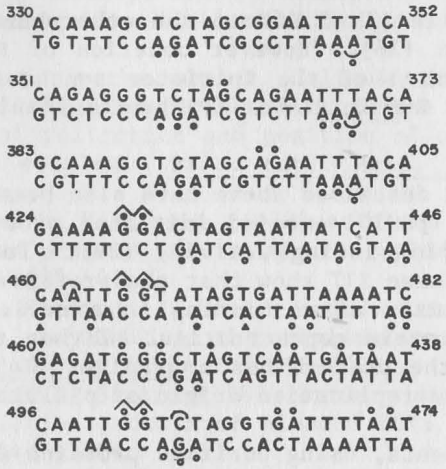


Fig. 13. Summary of the DNA-protein interaction studies between the pSC101 origin and the plasmid encoded 37.5-kd initiator protein. The sequences between the coordinates 496-474 and 460-482 constitute the nucleation site to which the protein binds at the lowest concentration. The sequences between 460-438 and 424-446 constitute the second binding domain. The sequence between 330-352 and the 3 direct repeat 5-330) constitute the remainder 3rd, 4th, and 5th binding sites. ●, strongly blocked from methylation; ○, weakly blocked; ▼, enhanced methylation; υ,η, enhanced DNase cleavage; ^, methylation interference.

The results presented here demonstrate that fusion of β-galactosidase does not affect the sequence-specific DNA-binding activities of the R6K and pSC101-encoded initiator proteins. This is not surprising in view of the fact that the DNA-binding domains of many proteins are located at a small region of the molecule and that the DNA-binding domain can be isolated without the loss of ligand binding activity by controlled proteolysis (23, 24). Previous work has shown that both the lac and the λ repressors can be fused to β-galactosidase without the loss of biological activities. Hence β-galactosidase fusions, in principle, could be used for rapid screening for sequence-specific DNA-binding proteins from a variety of systems.

The purified β-galactosidase tagged and nontagged proteins bind to 2 regions of the R6K chromosome. The binding Site I is located at or near ori γ but is separated by approximately 2,000 bp from ori α and 1,000 bp from ori β. The Site I is essential for DNA replication from all 3 origins (35). We have suggested that the binding Site I may serve as an "initiator loading site."

The binding Site II of R6K on the other hand is not essential for DNA replication (36). However deletion of this site leads to loss of autoregulation of the initiator synthesis (36). Previous work had shown that R6K initiator (Pi protein) autoregulated its own synthesis (35).

The techniques described above have also been used to partially purify the 37.5 kd pSC101-encoded initiator protein. This protein has sequence-specific binding activity (29). Footprinting experiments using exonuclease III show that the protein binds in a sequential manner to 4 sites after binding to a nucleation site at the lowest effective protein concentration. Either the binding is cooperative or else the protein has a gradient of affinities for the binding sites at the replication origin of pSC101.

Future experiments, using purified proteins and in vitro replication system, should reveal the role of the plasmid-encoded initiators in initiation of replication and plasmid incompatibility.

ACKNOWLEDGMENT

This work was supported by the grants AI-19881 and PO1 CA30246 awarded by the NIH. D. B. is an Established Investigator of the American Heart Association. We thank Hilda Smith for the preparation of this manuscript. The Fig. 6, 7, 11, 12, and 13 have been reproduced with the permission of the publishers of Cell (MIT Press).

REFERENCES

1. Kontomichalou, P., M. Mitani, and R. Clowes (1970) Circular R-factor molecules controlling penicillinase synthesis, replicating in Escherichia coli under either relaxed or stringent control. J. Bact. 104:34-44.
2. Cohen, S.N., and A.C.Y. Chang (1973) Recircularization and autonomous replication of a sheared R-factor DNA segment in Escherichia coli transformants. Proc. Natl. Acad. Sci., USA 70:1293-1297.
3. Cohen, S.N., and A.C.Y. Chang (1977) Revised interpretation of the origin of pSC101 plasmid. J. Bact. 132:734-737.
4. Crosa, J. (1980) Three origins of replication are active in vivo in the R plasmid RSF 1040. J. Biol. Chem. 255:11075-11077.
5. Inuzuka, N., M. Inuzuka, and D. Helinski (1980) Activity in vitro of three origins of replication of the antibiotic resistance plasmid RSF 1040. J. Biol. Chem. 255:11071-11074.
6. Shon, M., J. Germino, and D. Bastia (1982) The nucleotide sequence of the replication origin of the plasmid R6K. J. Biol. Chem. 257:13823-13827.

7. Crosa, J., L.K. Luttrop, and S. Falkow (1976) Mode of replication of the conjugative R plasmid RSF 1040 of E. coli. J. Bact. 129-454-466.
8. Lovett, M., R.A. Sparks, and D. Helinski (1975) Bidirectional replication of the plasmid R6K in E. coli: Correspondence between origin of relication and position of single strand break in relaxation complex. Proc. Natl. Acad. Sci., USA 72:2905-2909.
9. Kolter, R., and D. Henlinski (1978) Activity of the replication terminus of the plasmid R6K in hybrid replicons in E. coli. J. Mol. Biol. 124:425-441.
10. Bastia, D., J. Germino, J. Crosa, and J. Ram (1981) The nucleotide sequence surrounding the replication terminus of R6K. Proc. Natl. Acad. Sci., USA 78:2095-2099.
11. Germino, J., and D. Bastia (1982) Primary structure of the replication initiation protein of plasmid R6K. Proc. Natl. Acad. Sci., USA 79:5475-5479.
12. Stalker, D.M., R. Kolter, and D. Helinksi (1982) Plasmid R6K replication: Complete nucleotide sequence of an autonomously replicating segment. J. Mol. Biol. 161:33-43.
13. Kolter, R., M. Inuzuka, and D. Helinski (1978) Transcomplementation dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell 15:1199-1208.
14. Hasanuma, K., and M. Sakiguchi (1977) Replication of plasmid pSC101 in Escherichia coli K12: Requirement for dnaA function. Mol. Gen. Genet. 154:225-230.
15. Vocke, C., and D. Bastia (1983) Primary structure of the essential replicon of the plasmid pSC101. Proc. Natl. Acad. Sci., USA 80:6557-6561.
16. Churchward, G., P. Linder, and L. Caro (1983) The nucleotide sequence of replication and maintenance functions encoded by plasmid pSC101. Nucl. Acids Res. 11:5645-5659.
17. Meacock, P., and S.N. Cohen (1980) Partitioning of bacterial plasmids during cell division: A cis-acting locus that accomplishes stable plasmid maintenance. Cell 20:529-542.
18. Germino, J., J.G. Gray, H. Charbonneau, T. Vanaman, and D. Bastia (1983) Use of gene fusion and protein-protein interaction in the isolation of a biologically active regulatory protein: The replication initiator protein of plasmid R6K. Proc. Natl. Acad. Sci., USA 80:6848-6852.
19. Harper, E. (1970) Studies on the mechanism of action of bacterial collagenase. In Collagenase, I. Mandel, ed. Gordon and Breach, New York, pp. 19-24.
20. Germino, J., and D. Bastia (1984) Rapid purification of a cloned gene product by genetic fusion and site-specific proteolysis. Proc. Natl. Acad. Sci., USA Vol. 81 (in press).
21. Inuzuka, M., and D. Helinski (1978) Requirement of a plasmid-encoded protein for replication in vitro of plasmid R6K. Proc. Natl. Acad. Sci., USA 75:5381-5385.
22. Germino, J., and D. Bastia (1983) Interaction of the plasmid R6K-encoded replication initiator protein with its binding sites on DNA. Cell 34:125-134.

23. Ogata, R., and W. Gilbert (1978) An amino-terminal fragment of lac repressor binds specifically to lac operator. Proc. Natl. Acad. Sci., USA 75:5851-5854.
24. Pabo, C.O., W. Krovatin, A. Jeffrey, and R.T. Sauer (1982) The N-terminal arms of λ repressor wrap around the operator DNA. Nature (London) 298:441-443.
25. Riggs, A.D., H. Suzuki, and S. Bourgeoise (1970) Lac repressor operator interaction I: Equilibrium studies. J. Mol. Biol. 48:67-83.
26. Germino, J., and D. Bastia (1983) The replication initiator protein of plasmid R6K tagged with β-galactosidase shows sequence-specific DNA-binding. Cell 32:131-140.
27. Stalker, D.M., R. Kolter, and D. Helinski (1979) Nucleotide sequence of an origin of replication of the antibiotic resistance plasmid R6K. Proc. Natl. Acad. Sci., USA 76:1150-1154.
28. Miller, C.A., W.T. Tucker, F. Meacock, and S.N. Cohen (1983) Nucleotide sequence of the partition locus of pSC101. Gene 24:309-315.
29. Vocke, C., and D. Bastia (1983) DNA-protein interaction at the origin of DNA replication of the plasmid pSC101. Cell 35:495-502.
30. Vieira, J., and J. Messing (1982) The pUC plasmids, an M13 mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.
31. Lis, J.T., J.A. Simon, and C.A. Sutton (1983) New heat shock puffs and β-galactosidase activity resulting from transformation of Drosophila with an hep 70-lac Z hybrid gene. Cell 35:403-410.
32. Rose, M., M.J. Casadaban, and D. Botstein (1981) Yeast genes fused to β-galactosidase in Escherichia coli can be expressed normally in yeast. Proc. Natl. Acad. Sci., USA 78:2460-2464.
33. Nielsen, D.A., J. Chou, A. Mackrell, M.J. Casadaban, and D.F. Steiner (1983) Expression of a preproinsulin-β-galactosidase gene fusion in mammalian cells. Proc. Natl. Acad. Sci., USA 80:5198-5202.
34. Anglister, L., and I. Silman (1979) Molecular structure of elongated forms of electric eel acetylcholine sterase. J. Mol. Biol. 125:293-309.
35. Shafferman, A., R. Kolter, and D. Helinski (1982) Plasmid R6K DNA replication: Regulatory properties of the initiation protein. J. Mol. Biol. 161:67-72.
36. Germino, J. (1983) Studies on the initiation and termination of replication of plasmid R6K. Ph.D. thesis, Department of Microbiology, Duke University Medical Center.

POSITIVE REGULATION AND TRANSCRIPTION INITIATION OF XYL OPERONS ON TOL PLASMID

Teruko Nakazawa,* Sachiye Inouye, and Atsushi Nakazawa

*School of Allied Health Sciences
Yamaguchi University
and Department of Biochemistry
Yamaguchi University School of Medicine
Ube 755, Japan

INTRODUCTION

Degradative Pathway Specified by TOL

Members of the bacterial genus Pseudomonas have a capacity to degrade a wide range of organic compounds. Many degradative pathways are controlled by chromosomal genes, while some catabolic activities are specified by genes borne on plasmids.

A number of plasmids have been isolated which determine degradation of aromatic hydrocarbons and their products. These include NAH, encoding enzymes for naphthalene oxidation (1); SAL, controlling salicylate degradation (2); and TOL, specifying enzymes for degradation of toluene and xylenes (3,4). The plasmids carry a whole array of functional genes responsible for successive catabolism of substrates. Therefore, such plasmids would provide a useful experimental system for detailed analyses of genetic organization and of expression of catabolic pathways in nonenteric bacteria.

The natural host of TOL is Pseudomonas putida (arvilla) mt-2 (ATCC23973) that was isolated in 1959 by K. Hosokawa in Japan from enrichment culture of m-toluate. The reactions applied to toluene as encoded by the TOL plasmid are controlled in the 2 reaction sequences by 2 gene clusters (Fig. 1). The upper pathway sequence is responsible for oxidation of toluene to benzoate through benzyl alcohol and benzaldehyde. The lower pathway sequence includes the formation of catechol followed by the meta-cleavage of catechol and successive reactions to yield pyruvate and acetaldehyde. A whole

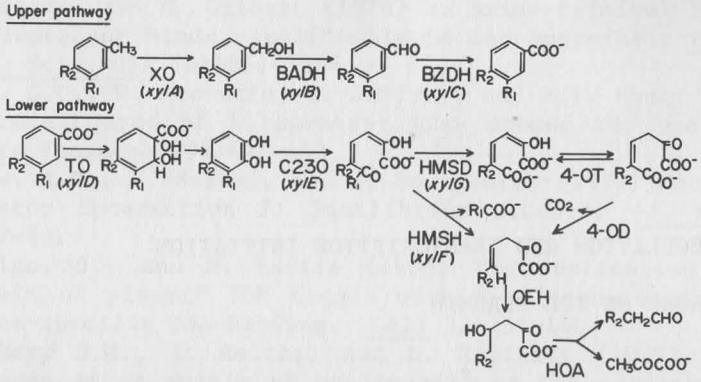


Fig. 1. The degradative pathway encoded by TOL. Enzyme abbreviations: XO, xylene oxygenase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate oxygenase; C230, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; 4-OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. xylA to xylG, genes for the degradative pathway. Compounds: R1, H; R2, H; toluene. R1, CH_3; R2, H; m-xylene. R1, H; R2, CH_3; p-xylene.

set of enzymes responsible for these reactions also catalyzes the metabolism of methylated derivatives of the aromatic compounds. The meta-fission of catechol is characteristic of plasmid-encoded aromatic catabolism of TOL, NAH, and SAL.

A Model for Regulation of Gene Expression

Based on the mutant analysis, Worsey et al. (5) proposed a model for the regulation of the early enzymes. The model is modified by recent studies (6,7) as shown in Fig. 2. The enzymes are organized in 2 regulatory blocks, i.e., operons. The first operon (xylABC) consists of xylA, xylB, and xylC for the upper pathway enzymes responsible for the conversion of aromatic hydrocarbons to carboxylic acids. The second operon (xylDEGF) consists of xylD, xylE, xylG, xylF and so forth for the lower pathway enzymes catalyzing further oxidation and complete degradation of carboxylic acids.

Two regulatory genes, xylR and xylS, are involved; xylR plays a role in the induction of both operons by the hydrocarbons and the alcohol metabolites, whereas xylS is responsible for the induction of only the second operon by the carboxylic acid. Both xylR and xylS are necessary for the induction of the second operon by m-xylene or m-methylbenzyl alcohol.

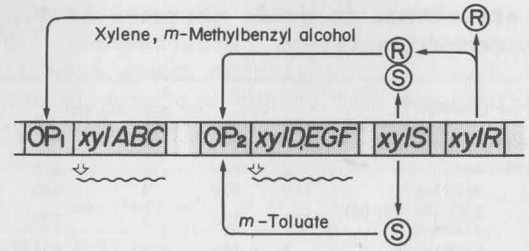


Fig. 2. A model for the positive regulation of xyl operons. The xylR product combines with the operator of xylABC (OP1) in the presence of m-xylene or m-methylbenzyl alcohol and activates the xylABC transcription. The xylS product combines with the operator of xylDEGF (OP2) in the presence of m-toluate and activates the xylDEGF transcription. Both xylR and xylS products are required for the m-xylene- or m-methylbenzyl alcohol-dependent activation of the xylDEGF transcription.

In the following sections, evidence will be presented that the xylR and xylS products are trans-acting in the xylABC and xylDEGF operons to activate the gene expression.

POSITIVE REGULATION OF XYL OPERONS

Isolation of RP4-TOL Recombinants

Since the Pseudomonas system for gene manipulation had not been established at the early stage of our work on TOL, we looked for a system that could be analyzed in E. coli.

The TOL plasmid in a strain of Pseudomonas aeruginosa PAO is unstable at high temperature, while a broad host-range IncP1 plasmid, RP4, is temperature-resistant (8). Plasmid RP4 (54-kb) was introduced into the PAO strain carrying TOL (117-kb), and clones were selected on m-toluate-M9 agar at 42°C. Thus, a recombinant plasmid pTN1 (114-kb) was obtained that was stable even at high temperature (9). The pTN1 plasmid in P. putida specified a constitutive low expression of meta-pathway enzymes, whereas the upper pathway enzymes remained inducible (Tab. 1). P. putida cells carrying pTN1 grew poorly on m-toluate-M9 agar and produced 2 types of segregants at a frequency of about 10^{-4} per cell (10). One of the segregants carried pTN2 (111-kb) that specified inducible synthesis of TOL enzymes similar to that of the wild-type TOL plasmid. Another segregant carried pTN8 (114-kb) which specified a constitutive high level of the meta-pathway enzymes (Tab. 1).

Tab. 1. Enzyme activities in crude extracts of P. putida carrying RP4-TOL recombinants.

Strain	Inducer[a]	Sp act (mU/mg) BADH	C23O	HMSH	β-Lactamase[b]
TN2100(pTN1)	None	2	240	3	410
	m-Xylene	550	200	4	480
	m-Methylbenzyl alcohol	110	250	3	370
	m-Toluate	2	250	3	420
TN2100(pTN2)	None	2	270	3	420
	m-Xylene	350	8900	120	400
	m-Methylbenzyl alcohol	70	7800	80	340
	m-Toluate	1	8000	90	470
TN2100(pTN8)	None	4	2340	23	460
	m-Xylene	520	1950	22	410
	m-Methylbenzyl alcohol	330	2200	21	490
	m-Toluate	3	2410	22	430

[a] Cells were grown at 27°C overnight in L broth in the absence or presence of m-xylene vapor, 5mM m-methylbenzyl alcohol, or 5 mM m-toluate.

[b] β-Lactamase encoded by RP4 was determined to check the copy number of recombinants.

Inefficient and Temperature-sensitive Expression of xyl Operons in E. coli

RP4-TOL recombinants were transferred to E. coli, and the expression of xyl genes in E. coli was determined at various temperatures (Tab. 2). C230 and HMSH levels in cells of E. coli carrying either pTN1, pTN2, or pTN8 at 30°C are substantially lower than those in P. putida grown at 27°C (Tab. 1) under both induced and noninduced conditions. Induced levels of BADH are slightly lower in E. coli than in P. putida. The constitutive expression of C230 and HMSH encoded by pTN1 and pTN8 in E. coli is temperature resistant, while the inducible synthesis of BADH encoded by pTN1, pTN2, and TN8 and the induction of C230 and HMSH encoded by pTN2 is temperature sensitive. The thermosensitivity of enzyme induction was also observed in P. aeruginosa.

Structure of RP4-TOL Recombinants

Plasmid pTN2 has the entire length of RP4 and a part of TOL (56-kb) that contains all the degradative genes as well as the regulatory genes required for enzyme induction (10). The physical map of the TOL region of pTN2 (Fig. 3) is indistinguishable from the corresponding region of TOL (pWW0) (11,12). The structural genes of the operons were mapped on pTN2 by using the molecular cloning technique in E. coli (13,14). The genetic map of pTN2 is consistent

Tab 2. Enzyme activities in crude extracts of E. coli carrying RP4-TOL recombinants grown at various temperatures.[a] Cells were grown overnight in L broth in the absence or presence of m-xylene vapor, 5mM m-methylbenzyl alcohol, or 5mM m-toluate.

Strain	Temp.	Inducer[a]	Sp act (mU/mg) BADH	C23O	HMSH	β-Lactamase
20SO(pTN1)	30°C	None	10	9	0.1	1390
		m-Xylene	119	3	<0.1	790
		m-Methylbenzyl alcohol	62	5	<0.1	1380
		m-Toluate	9	7	0.1	1470
	37°C	None	8	29	0.3	1420
		m-Methylbenzyl alcohol	22	28	0.3	1590
		m-Toluate	13	37	0.6	1620
	42°C	None	21	110	0.5	450
		m-Methylbenzyl alcohol	18	110	0.6	400
		m-Toluate	31	120	0.7	430
20SO(pTN2)	30°C	None	10	6	<0.1	1910
		m-Xylene	215	1670	14.8	1640
		m-Methylbenzyl alcohol	60	750	7.4	1670
		m-Toluate	12	150	2.1	1620
	37°C	None	13	3	<0.1	1950
		m-Methylbenzyl alcohol	24	290	2.9	2160
		m-Toluate	15	61	0.7	2110
	42°C	None	27	3	<0.1	550
		m-Methylbenzyl alcohol	24	5	<0.1	670
		m-Toluate	31	6	<0.1	690
20SO(pTN8)	30°C	None	6	320	0.1	1890
		m-Methylbenzyl alcohol	60	300	3.0	1890
		m-Toluate	11	310	2.8	2000
	37°C	None	12	630	3.6	1510
		m-Methylbenzyl alcohol	19	830	5.0	1700
		m-Toluate	13	630	4.5	1940
	42°C	None	20	740	6.9	640
		m-Methylbenzyl alcohol	14	810	6.3	570
		m-Toluate	21	760	6.0	560

[a] Cells were grown overnight in L broth in the absence or presence of m-xylene vapor, 5mM m-methylbenzyl alcohol, or 5mM m-toluate.

with those for pWW0 mapped by transposon mutagenesis, molecular cloning, and analysis of insertion mutants (7,12,15). We have determined the complete nucleotide sequence of xylE (921 bp) and its flanking sequences (16). The xylE sequence of pWW0 reported by Zukowski et al. (17) is coincided well but not identical to that of pTN2.

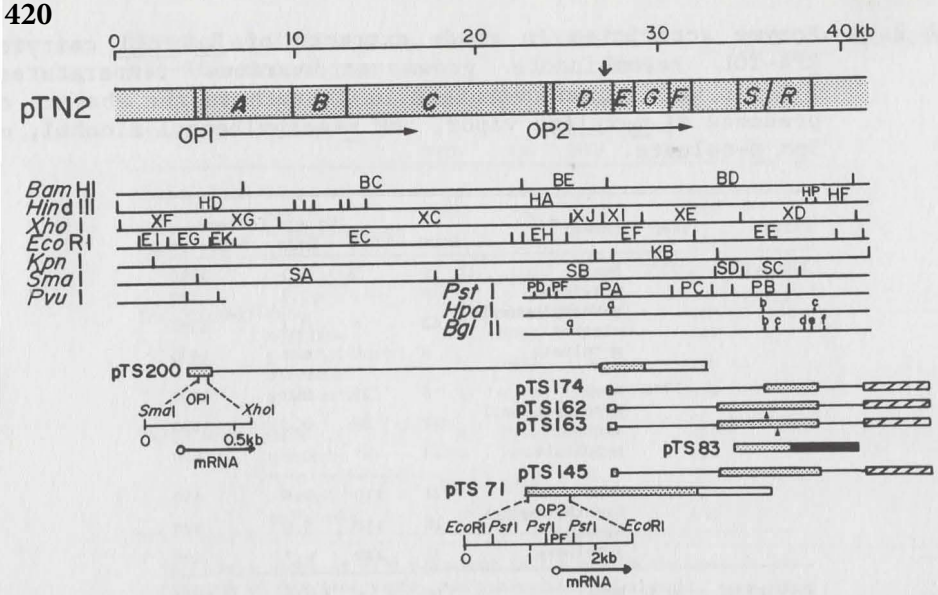


Fig. 3. Physical and functional map of the TOL region of pTN2. The notations of xyl genes and operator-promoter regions are the same as described in the legend to Fig. 2. A vertical arrow indicates the insertion site of a 3.6-kb segment in pTN1. The horizontal arrows indicate the direction of transcription. The lower part of the figure shows the structures of hybrid plasmids. Stippled, open, hatched, and closed boxes indicate the TOL, pBR322, pACYC 184, and pACYC177 regions, respectively. The fine mapping of transcription start sites in OP1 and OP2 are also indicated.

In addition to pTN2, several recombinants of a IncP1 plasmid and TOL were reported (18-22). A transposition model involving a unique segment of the TOL plasmid is unlikely, since the TOL moieties present in the different RP4-TOL plasmids are not identical (21). The pTN1 plasmid has an insertion of 3.6-kb fragment at the right end of the BamHI BE fragment in Fig. 3, which leads a constitutive low expression of xylE, xylG, and xylF.

During in vivo formation of pTN1 in P. aeruginosa, 2 events should have occurred at relatively high frequencies. One was the insertion of the 3.6-kb segment, and the other was the recombination of the 56-kb TOL segment and the temperature-resistant RP4 replicon. Thus, the cells carrying pTN1 could grow on m-toluate at high temperature because of the temperature-resistant replication and expression. Although the xylD expression was inducible and temperature-sensitive in pTN1, the chromosomal isofunctional enzyme, benzoate oxygenase might be able to complement the activity.

The 3.6-kb insertion found in pTN1 is very similar to an insertion (3.4-kb) described by Lehrbach et al. (15). Both insertions lead to inactivation of TOL genes and have no restriction sites for HindIII, EcoRI, SalI, PvuII, XhoI, PstI, BamHI, and BglII. The parent plasmid used to obtain insertion mutants by Lehrbach et al. is an RP4-TOL plasmid, pED3300, containing a 69-kb segment of TOL. From restriction, DNA hybridization, or heteroduplex analysis, the segment was shown to originate from within pWWO fragment HindIII-E (15) that is involved in the 69-kb TOL segment of pED3300, but not in the 56-kb segment of pTN2. The 3.6-kb insert can excise precisely from pTN1 to produce pTN2 and also can cause deletions of nearby DNA segments (10).

Plasmid pTN8 is a spontaneous mutant of pTN1 which was found in a segregant selected as a good grower on m-toluate. C230 is synthesized constitutively at a high level in cells carrying pTN8 (Tab. 1 and 2), and the plasmid still contains the 3.6-kb insert at the same site and orientation as in pTN1. It appears that the DNA sequence of 3.6-kb insert in pTN1 changed to produce a strong promoter in pTN8. We are currently investigating the DNA sequence surrounding the transcription start site within the insertion of pTN8.

Molecular Cloning of Operator-Promoter Regions of xyl Operons and Regulatory Genes xylR and xylS

As described in a previous section, pTN2 in E. coli specifies inducible synthesis of TOL enzymes similar to that in P. putida. These observations prompted us to analyze the regulation of xyl expression by molecular cloning in E. coli.

The lower part of Fig. 3 shows the structures of hybrid plasmids constructed to map the regulatory region of the xylABC and the xylDEGF operons as well as the regulatory genes xylR and xylS (6,14).

Plasmid pTS200 has a 1.7-kb PvuI-EcoRI fragment (OP1) joined to XhoI fragment XI (xylE) on pBR322 replicon. Plasmid pTS174 contains a 2.7-kb HpaI fragment (xylR) on pACYC184 replicon, pTS83 contains the PstI-PB fragment (xylS) on pACYC177 replicon, and pTS145 contains a 5.4-kb KpnI-HindIII fragment (xylR and xylS). The HindIII fragment HP is contained in PTS145. Plasmids pTS162 and pTS163 are insertion derivatives of pTS145 that were constructed by insertion of the 1.5-kb kan fragment of Tn5 into the BglII b site and c site, respectively. Plasmid pTS71 is a TOL-pBR322 hybrid containing xylD, xylE, xylG and xylF (13).

The activities of C230 in cells carrying these plasmids are shown in Fig. 4 (6). Cells carrying pTS200 alone do not show induction of the enzyme. In the presence of either pTS174 or pTS162, the enzyme is induced by m-xylene or m-methylbenzyl alcohol. pTS163 has

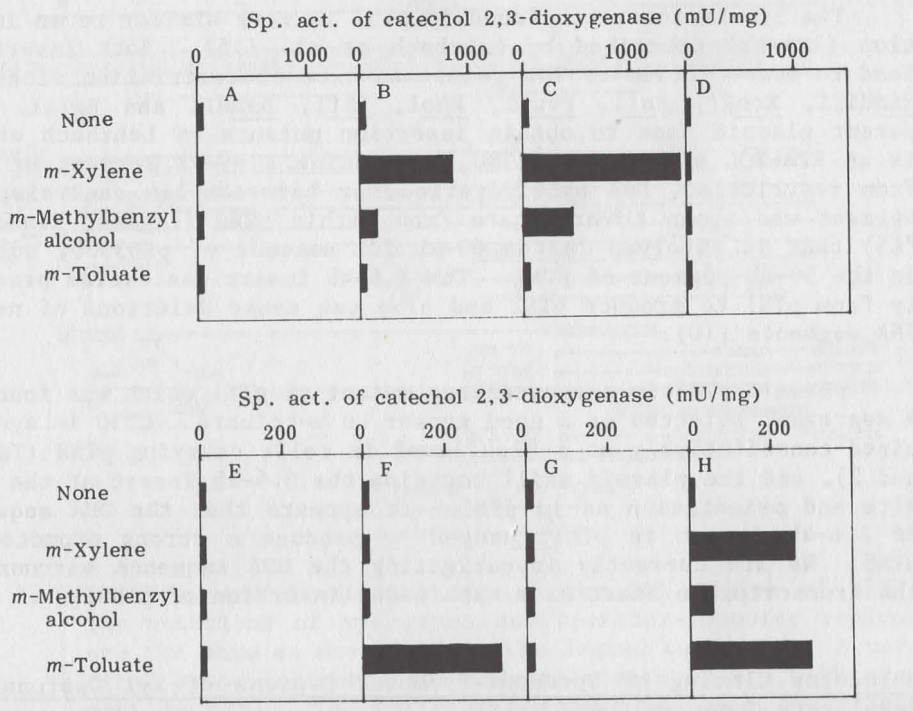


Fig. 4. Activation of xylABC and xylDEGF operons by xylR and xylS. Cells of E. coli carrying hybrid plasmids were grown in L broth at 30°C in the absence (none) or presence of m-xylene, m-methylbenzyl alcohol, or m-toluate. Plasmids carried: A, pTS200 (OP1-$xylE^+$); B, pTS200 and pTS174 ($xylR^+$); C, pTS200 and pTS162 ($xylR^+$); D, pTS200 and pTS163 ($xylS^+$); E, pTS71 (OP2-$xylDEGF^+$); F, pTS71 and pTS83 ($xylS^+$); G, pTS71 and pTS174; H, pTS71 and pTS145 ($xylR^+$ $xylS^+$).

no effect on the activation of OP1. These results indicate that pTS200 contains OP1 and that the xylABC operon is activated by the gene product of xylR that is present in the 2.8-kb region from the BglII b site to the HpaI c site. OP2 was further mapped on the 0.6-kb fragment ranging from the left end of the SmaI fragment SA to the right end of the XhoI fragment XF (6).

Cells carrying pTS71 and either pTS83 or pTS145 show induction of C230 in the presence of m-toluate, while pTS174 has no effect on the induction. In addition, pTS163 but not pTS162 gave m-toluate -dependent induction. Therefore, pTS71 contains OP2, and pTS83, pTS145 and pTS163 contain xylS that was mapped on the PstI PB fragment (Fig. 3). OP2 was further analyzed and mapped on the PstI PF fragment (Fig. 3) (6).

The m-xylene- or m-methylbenzyl alcohol-dependent induction of C230 encoded by pTS71 occurs only in the presence of pTS145. As evidenced by the activation of OP1, both pTS174 and pTS162 contain the functional xylR gene. If the same xylR gene is involved in the activation of both operons, the xylR gene alone is not sufficient for the induction of xylDEGF. It was concluded that both xylR and xylS are essential to the m-xylene- or m-methylbenzyl alcohol-dependent activation of xylDEGF. The same conclusion was reached by Franklin et al. from the analysis of transposon mutations (7).

In the above experiments, the operator-promoters were cloned on the pBR322 replicon, and the regulatory genes were cloned on either the pACYC184 or pACYC177 replicon. Since pBR322 is compatible with pACYC replicon, the trans-acting effect of xylR or xylS could be determined.

In summary, we have analyzed the regulation of xyl gene expression by molecular cloning in E. coli, and the results obtained so far are consistent with the positive-regulation model shown in Fig. 2.

The xylE Gene Product C230 as a Promoter Probe

The precise mapping of OP1 and OP2 as well as xylR and xylS described above was carried out by monitoring the expression of xylE. The xylE product, C230 catalyzes the conversion of catechol to a yellow compound, 2-hydroxymuconic semialdehyde which has an absorption peak at 375 nm with the molar extinction coefficient of 14,700 at pH7.5 (23). The activity within cells is easily detected by spraying 0.1 M catechol solution on colonies and observing development of a yellow color. Catechol may be transported into cells by a simple diffusion and attacked by C230 which has a low Km value for catechol (3 μM) (23).

C230 has the molecular weight of 140,000 and consists of 4 identical subunits consisting of 307 amino acid residues (16). Several base pairs upstream from the initiation codon, the ribosome binding site has been predicted, that is complementary to the 16S rRNA of E. coli and P. aeruginosa (24). There are stop codons in front of the initiation codon so that a fused product of xylE is not synthesized upon ligation with the coding sequence upstream from the gene. In addition, since xylE is neither the first nor the last cistron of the operon, there is no proper promoter or terminator around the gene.

When xylE was joined to the tetracycline promoter of pBR322 by inserting the BamHI-BD fragment into the BamHI site, C230 was synthesized by a read-through transcription from the vector promoter in E. coli that was more than 300-fold of that encoded by pTN2 under noninduced conditions (13). Therefore, the low expression of TOL genes in E. coli is not due to a translational barrier, but is a reflection of poor transcription efficiency.

The gene expression of colicin E1 was studied by following the induction of C230 encoded by a ColE1-*xylE* fusion plasmid (25). The operator-promoter region of the colicin E1 gene was joined to the SstII fragment (1.2-kb) containing *xylE*. It was found that the C230 synthesis from the hybrid plasmid was cyclic AMP-dependent and controlled by the *recA-lexA* system (25). The *xylE* gene, therefore, is a useful probe for detection and quantitative determination of promoters in a wide range of gram-negative bacteria.

The usefulness of *xylE* as a promoter probe was also demonstrated in a *Bacillus subtilis* system (17).

TRANSCRIPTION INITIATION OF *XYL* OPERONS

Localization of Transcription Start Site of *xylABC* Operon

The induction pattern of *xyl* operons in *E. coli* is quite similar to that in *P. putida*, suggesting that the same operator-promoter is functioning in both bacteria. To see whether this is the case, we determined the in vivo transcription start site of the *xylABC* operon by S1 nuclease and reverse transcriptase mapping (26).

The detailed mapping revealed that the OP1 region is in the 0.6-kb fragment ranging from the left end of the SmaI fragment SA to the right end of the XhoI fragment XF (Fig. 3) (6). A fragment containing the OP1 region was labeled at the XhoI site of the sense strand and used as a probe for S1 mapping analysis that was performed as described (27). Crude RNA preparations were obtained from cells of *P. putida* or *E. coli* carrying pTN2 grown under induced or noninduced conditions. RNA was hybridized with the probe DNA, treated with S1 nuclease, and the protected fragments were subjected to polyacrylamide-urea gel. It was found that the transcription started at 390 nucleotides upstream from the labeled XhoI site when RNA from *m*-methylbenzyl alcohol-induced cells of *P. putida* carrying pTN2 was subjected to the assay.

The transcription start site was further analyzed by reverse transcriptase mapping (28). The sense strand of a small DNA fragment was labeled at the 5' ends, hybridized with mRNA and extended with reverse transcriptase to the transcription start site. The extended product was analyzed by coelectrophoresis on a sequence gel with chemically cleaved reaction products by the procedure of Maxam and Gilbert. As a result, a single start site was found with RNA from induced cells of *P. putida* carrying pTN2. The same start site was also found with RNA from induced cells of *E. coli* carrying pTN2, but the amount of mRNA was only 10% of that of *P. putida*. The mRNA levels in these bacteria determined by S1 mapping coincided with the enzyme levels in vivo (see Tab. 1 and 2). No transcription was found with RNA preparations from noninduced cells of *P. putida* or *E. coli* carrying pTN2 or cells free from the plasmid.

These results indicate that the *m*-methylbenzyl alcohol-dependent transcription of the *xylABC* operon in *E. coli* starts at exactly the same site as in *P. putida*. The low level of mRNA in *E. coli* appears to be a reflection of inefficient transcription of *xyABC* by *E. coli* RNA polymerase.

Localization of Transcription Start Site of *xylDEGF* Operon

The transcription start site of *xylDEGF* was also determined by S1 nuclease and reverse-transcriptase mapping (29). We have previously mapped OP2 on the PF fragment (Fig. 3) (6). S1 nuclease mapping revealed that the transcription started in the PF fragment in the induced cells of *P. putida* or *E. coli* carrying pTN2.

The precise initiation site for transcription of *xylDEGF* was further determined by reverse-transcriptase mapping. As for the *xylABC* operon, a single start site was found in both *P. putida* and *E. coli* cells under both *m*-methylbenzyl alcohol- and *m*-toluate-induced conditions.

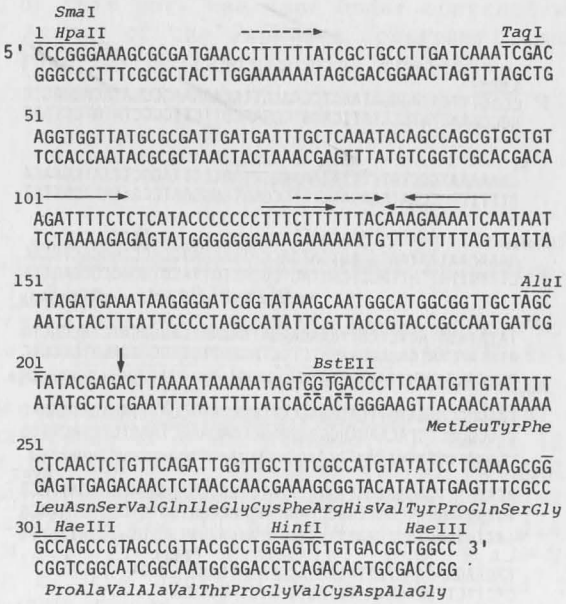


Fig. 5. Nucleotide sequence of the OP1 region of the TOL plasmid (26). Nucleotides are numbered from the SmaI site. A vertical arrow indicates the transcription start site of the *xylABC* operon. Inverted repeats are indicated by solid horizontal arrows and direct repeats, by dashed horizontal arrows. The underlined bases are complementary to the 3' end of the 16S rRNA of *P. aeruginosa*. Amino acids predicted from the nucleotide sequence are shown in italics.

The transcription in E. coli cells was poor especially when induced by m-toluate. It is not known whether m-toluate uptake in E. coli is lower than in P. putida.

Nucleotide Sequence of OP1 and OP2

The sequences surrounding the transcription start sites of xylABC (340 bp) (Fig. 5) and xylDEGF (336 bp) (Fig. 6) were determined (26) by the Maxam and Gilbert procedure.

In the region downstream from the transcription start site, there is one AUG in xylABC and 3 AUGs in xylDEGF, from which a continuous reading frame exists in each sequence at least up to the end of the sequences determined. The predicted amino acid sequences probably correspond to the NH_2-terminal region of the enzyme of the first cistron of each operon. Several base pairs upstream from the initiation codon of each operon, there is a typical ribosome binding site.

Preceding the transcription initiation site of xylABC, there are two pallindromic AT-rich sequences as candidates for operators,



Fig. 6. Nucleotide sequence of the OP2 region of the TOL plasmid (29). Nucleotides are numbered from the HpaI site. A vertical arrow indicates the transcription start site of the xylDEGF operon. An inverted repeat is indicated by horizontal arrows. The underlined bases are complementary to the 3' end of the 16S rRNA of P. aeruginosa. Amino acids predicted from the nucleotide sequence are shown in italics.

which have the axis of diad symmetry at -85 and -76 bp (Fig. 5). Such a sequence, however, is absent from OP2. On the other hand, a pallindromic sequence is found at -15 bp (Fig. 6). The xylABC operon is activated by xylR, whereas the xylDEGF operon is activated either by xylS alone or xylS together with xylR. Thus, the sequences of the 2 operators could be different from each other.

In the region upstream from the transcription start sites in both sequences, the consensus sequences of E. coli promoters were not found with either the -10 region (Pribnow box) or the -35 region (RNA polymerase recognition site). The sequences of the 2 operons, however, are homologous with each other around at the -35 and -10 regions. The homologous sequences may represent signals for transcription initiation of positively controlled operons in Pseudomonas.

ACKNOWLEDGMENTS

This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan. A part of this work was done under contract with the Science and Technology Agency of the Japanese Government, and was supported by the Special Fund for Multiministrial Projects.

REFERENCES

1. Dunn, N.W., and I.C. Gunsalus (1973) Transmissible plasmid coding early enzymes of naphthalene oxidation in Pseudomonas putida. J. Bact. 144:974-979.
2. Chakrabarty, A.M. (1972) Genetic basis of the biodegradation of salicylate in Pseudomonas. J. Bact. 112:815-823.
3. Williams, P.A., and K. Murray (1974) Metabolism of benzoate and methylbenzoates by Pseudomonas putida (arvilla) mt-2: Evidence for the existence of a TOL plasmid. J. Bact. 120:415-423.
4. Worsey, M.J., and P.A. Williams (1975) Metabolism of toluene and xylenes by Pseudomonas putida (arvilla) mt-2: Evidence for a new function of the TOL plasmid. J. Bact. 124:7-13.
5. Worsey, M.J., F.C.H. Franklin, and P.A. Williams (1978) Regulation of the degradative pathway coded for by the TOL plasmid (pWWO) for Pseudomonas putida mt-2. J. Bact. 134:757-764.
6. Inouye, S., A. Nakazawa, and T. Nakazawa (1983) Molecular cloning of the regulatory gene xylR and the operator-promoter regions of xylABC and xylDEGF operons of TOL plasmid. J. Bact. 155:1192-1199.
7. Franklin, F.C.H., P.R. Lehrbach, R. Lurz, B. Rueckert, M. Bagdasarian, and K.N. Timmis (1983) Localization and functional analysis of transposon mutations in regulatory genes of the TOL catabolic pathway. J. Bact. 154:676-685.

3. Nakazawa. T. (1978) TOL plasmid in Pseudomonas aeruginosa PAO: thermosensitivity of self-maintenance and inhibition of host cell growth. J. Bact. 133:527-535.
9. Nakazawa, T., E. Hayashi, T. Yokota, T. Ebina, and A. Nakazawa (1978) Isolation of TOL and RP4 recombinants by integrative suppression. J. Bact. 134:270-277.
0. Nakazawa, T., S. Inouye, and A. Nakazawa (1980) Physical and functional mapping of RP4-TOL plasmid recombinants: Analysis of insertion and deletion mutants. J. Bact. 144:222-231.
1. Downing, R., and P. Broda (1979) A cleavage map of the TOL plasmid of Pseudomonas putida mt-2. Mol. Gen. Genet. 177:189-191.
2. Franklin, F.C.H., M. Bagdasarian, M.M. Bagdasarian, and K.N. Timmis (1981) Molecular and functional analysis of the TOL plasmid pWW0 from Pseudomonas putida and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci., USA 78:7458-7462.
3. Inouye, S., A. Nakazawa, and T. Nakazawa (1981) Molecular cloning of TOL genes xylB and xylE in Escherichia coli. J. Bact. 145:1137-1143.
4. Inouye, S., A. Nakazawa, and T. Nakazawa (1981) Molecular cloning of gene xylS of the TOL plasmid: Evidence for positive regulation of the xylDEGF operon by xylS. J. Bact. 148:413-418.
5. Lehrbach, P.R., D.J. Jeenes, and P. Broda (1983) Characterization by molecular cloning of insertion mutants in TOL catabolic functions. Plasmid 9:112-125.
6. Nakai, C., H. Kagamiyama, M. Nozaki, T. Nakazawa, S. Inouye, Y. Ebina, and A. Nakazawa (1983) Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of Pseudomonas putida mt-2. J. Biol. Chem. 258:2923-2928.
7. Zukowski, M.M., F.F. Gaffney, D. Speck, M. Kauffmann, A. Findeli, A. Wisecup, and J.P. Lecocq (1983) Chromogenic identification of genetic regulatory signals in Bacillus subtilis based on expression of cloned Pseudomonas genes. Proc. Natl. Acad. Sci., USA 80:1101-1105.
8. Jacoby, G.A., J.E. Rogers, A.E. Jacob, and R.W. Hedges (1978) Transposition of toluene degrading genes and expression in E. coli. Nature 274:179-180.
9. Chakrabarty, A.M., D.A. Friello, and L.H. Bopp (1978) Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various organisms. Proc. Natl. Acad. Sci., USA 75:3109-3122.
20. White, G.P., and N.W. Dunn (1977) Apparent fusion of the TOL plasmid with the R91 drug resistance plasmid in Pseudomonas aeruginosa. Aust. J. Biol. Sci. 30:345-355.
21. Muelien, P., R.G. Downing, and P. Broda (1981) Excision of the 40-kb segment of the TOL plasmid from P. putida mt-2 involves direct repeats. Mol. Gen. Genet. 184:97-101.
22. Jeenes, D.J., and P.A. Williams (1982) Excision and integration of the degradative pathway genes from the TOL plasmid pWW0. J. Bact. 150:188-194.

23. Nozaki, M. (1979) Oxygenases and dioxygenases. Topics of Current Chemistry. 78:145-186.
24. Shine, J., and L. Dalgarno (1975) Determination of cistron specificity in bacterial ribosomes. Nature (Lond.) 254:34-38.
25. Ebina, Y., Y. Takahara, K. Shirabe, M. Yamada, T. Nakazawa, and A. Nakazawa (1983) Plasmid encoded regulation of colicin E1 gene expression. J. Bact. 156:487-492.
26. Inouye, S., Y. Ebina, A. Nakazawa, and T. Nakazawa (1984) Nucleotide sequence surrounding transcription initiation site of xylABC operon on TOL plasmid of Pseudomonas putida. Proc. Natl. Acad. Sci., USA 81:1688-1691.
27. Ebina, Y., and A. Nakazawa (1983) Cyclic AMP-dependent initiation and ρ-dependent termination of colicin E1 gene transcription. J. Biol. Chem. 258:7072-7078.
28. Débarbouillé, M., and O. Raiboud (1983) Expression of the Escherichia coli malPQ operon remains unaffected after drastic alteration of its promoter. J. Bact. 153:1221-1227.
29. Inouye, S., A. Nakazawa, and T. Nakazawa (1984) Nucleotide sequence of the operator-promoter region of the xylDEGF operon on TOL plasmid in Pseudomonas putida. Gene 29:323-330.

CHAIRMEN'S INTRODUCTION: PLASMID TRANSFER

Neil Willetts and Don B. Clewell[1]

Biotechnology Australia Pty. Ltd.
Roseville, NSW 2069, Australia

Bacterial plasmids from a wide range of genera are now being studied in numerous laboratories around the world, and from a variety of perspectives. Plasmids provide systems for the study of DNA replication and its control; they are useful in studies of bacterial genetics and are invaluable as vectors for molecular cloning. From the standpoint of the bacteria, however, what is it about these molecules that makes them so ubiquitous?

It now seems clear that plasmids provide bacterial populations with a greatly enhanced flexibility for rapid change. Bacteria are among the most variable organisms in nature, and the role of plasmids in this capacity is of major importance. One of the most significant features of plasmids is their ability to transfer from one bacterium to another. This most frequently occurs by a conjugative process, although in some situations plasmids can transfer by transduction or transformation. The prevalance of conjugation as a plasmid phenotype is perhaps an indication of its importance.

While many plasmids are conjugative, others can transfer only if mobilized by a conjugative plasmid. Such nonconjugative plasmids often supply genes contributing to the mobilization process, while others may need to be co-integrated with the mobilizing plasmid; even the bacterial chromosome can be transferred by the latter mechanism. Cointegrate structures are frequently intermediates in the transposition of an insertion sequence or transposon from one replicon to another.

---

[1]Department of Oral Biology and Microbiology/Immunology, Schools of Dentistry and Medicine and The Dental Research Institute, The University of Michigan, Ann Arbor, Michigan 48109.

The ultimate advantage of the resultant DNA mobility is that when a given bacterial cell has a gene that is uniquely protective or provides a growth advantage in a certain environment, a mechanism is available for distributing this information to other members of the bacterial population. As the information is gained and more cells become potential donors, the speed at which the gene can spread through the population increases; and selective processes aid its establishment.

Recent studies of conjugation in a number of different systems has revealed a rich variety of means whereby DNA transfer can be accomplished. The formation of mating pairs in gram-negative bacteria via an extracellular organelle, the pilus, can be contrasted to the pheromone-induced aggregation of certain gram-positive species; and the 33-kb DNA segment required for conjugation by IncF plasmids is distinguished from the 2 kb required by some Streptomyces plasmids. In the papers which follow, we shall learn more of this diversity.

LINEAR PLASMIDS WITH TERMINAL INVERTED REPEATS OBTAINED FROM STREPTOMYCES ROCHEI AND KLUYVEROMYCES LACTIS

Kenji Sakaguchi, Hirohiko Hirochika, and Norio Gunge

Mitsubishi-Kasei Institute of Life Sciences
Minamiooya 11, Machida, Tokyo, Japan, 194

SUMMARY

Linear plasmids with inverted terminal repeats of 614 bp were obtained from Streptomyces rochei which produced lankacidin. The 5' ends were blocked by the association of a terminal protein. A DNA model of racket frame-like structure is presented implying the juxtaposition of 2 double-stranded DNAs of the same sequence through the binding of cohesive proteins which recognize and bind to the DNA.

Two linear plasmids with the inverted terminal repeats of 202 and 184 bp were obtained from a yeast, Kluyveromyces lactis. A killer toxin was produced from the shorter plasmid. The protein toxin inhibited the adenylatecyclase activity of the yeast membrane.

INTRODUCTION

Most procaryotic and eucaryotic plasmids reported are composed of covalently-closed circular DNAs. Linear plasmids were first found in our laboratory from microbes. From Steptomyces rochei producing the antibiotics lankacidin and lankamycin, 2 linear plasmids of 17 kb designated as pSLA 1 and pSLA 2 (1,2,3,4) were obtained in 60 copies each per chromosome, as well as a circular plasmid, pSLA 3, which had the abilities of self-transmissibility and pock formation when the strain was costreaked with other strains.

From the yeast Kluyveromyces lactis which grows in cheese whey utilizing lactose as a carbon source, 2 linear plasmids of different

Tab. 1. Actinomycetes strains tested for the presence of plasmid.

Antibiotics or β-lactamase produced	Strain	Antibiotics or β-lactamase produced	Strain
Blasticidin S	*Streptomyces griseochromogenes* 2A-327	Streptothricin	*Streptomyces lavendulae* NIHJ-E-2
			Streptomyces microflavus NIHJ-13-A
Lankacidin	*Streptomyces* sp. 6642-GC$_1$	Chlortetra-cycline	*Streptomyces aureofaciens* NRRL-B-2209
	Streptomyces sp. 7434-AN$_4$		
Lonomycin	*Streptomyces ribosidificus* TM-481	Polyoxin	*Streptomyces cacaoi* var. *asoensis* POM-23-1
Viomycin	*Streptomyces diastatochromogenes* KCC-S-0119	Polyoxin + lysocellin	*Streptomyces cacaoi* var. *asoensis* POM-23-1 mutant
	Streptomyces vinaceus ICC-S-0091	Methylsalino-mycin	*Streptomyces albus* ATCC-21838
	Streptomyces californicus KCC-S-0143		
	Streptomyces puniceus KCC-S-0406	Xanthomycin	*Streptomyces* sp. 1134-7
	Streptomyces sp. KCC-U-0197	Geldanamycin	*Streptomyces hygroscopicus* 434
	Streptoverticillium olivoreticuli KCC-S-0197	Cephamycin	*Streptomyces clavuligerus* KCC-X-0710
			Streptomyces clavuligerus IFO 13307
Mikamycin	*Streptomyces mitakaensis* 74-4		*Streptomyces lipmanii* KCC-S-0711
Cycloheximide	*Streptomyces* sp. 2217-G$_1$		*Streptomyces lipmanii* IFO 13306
	Streptomyces sp. 8465-MC$_1$		*Streptomyces rochei* IFO 12908
	Streptomyces sp. 8482-CC$_1$		*Streptomyces fimbriatus* KCC-S-0190
Neomycin	*Streptomyces albogriseolus* NRRL-B-1305	Gentamicin	*Micromonospora echinospora* NRRL-2985
	Streptomyces sp. 7068-CC$_1$	β-Lactamase	*Streptomyces* sp. E-750-3
	Streptomyces sp. 8104-MC$_2$		*Streptomyces* sp. E-756-1

sizes were found in 50-100 copies each per haploid cell. They were designated as pGK1 1 and pGK1 2; 8, 9 kb and 13, 4 kb, respectively (5,6).

A protein toxin against Saccharomyces, Kluyveromyces, and several other yeast species was produced from the shorter plasmid, pGK1 1. The pGK1 1 DNA was totally sequenced and it contained 4 open reading frames.

The existence of linear plasmids in maize (Zea mays) mitochondria has been reported by Pring et al. (7). Their nucleotide sequences with inverted terminal repeats were recently reported (8) to possess a DNA structure similar to our Streptomyces and Kluyveromyces plasmids. From a fungus, Ascobolus inversus, a linear plasmid was found by Francou (9) which may have possessed the inverted terminal repeats.

LINEAR PLASMIDS FROM STREPTOMYCES ROCHEI

Isolation of Circular and Linear DNA Plasmids from Various Streptomyces Species

Thirty-four Streptomyces strains producing various antibiotics,

Tab. 2. Summary of plasmids from four strains of Streptomyces.

Strain	Antibiotics produced	Plasmid	S-value	Mol.Wt. (×10^{-6})
S. puniceus KCC-S-0406	viomycin	pSPU1	45.8	17.3
		pSPU2	69.0	39.1
Streptomyces sp. 2217-G$_1$	cycloheximide	pSCY1	21.9	3.2
		pSCY2	46.7	18.0
Streptomyces sp. 7068-CC$_1$	neomycin	pSNE1	51.7	22.2
		pSNE2	75.9	47.0
S. hygroscopicus 434	geldanamycin	pSHY1	67.9	37.9

including viomycin, blasticidin-S, cycloheximide, cephamycin, and others were isolated and generously supplied by Professor N. Otake, Institute of Applied Microbiology, University of Tokyo (Tab. 1 and 2). Through CsCl-EtBr gradient centrifugation, 7 circular plasmids were isolated from 4 strains of Streptomyces. The plasmids obtained from cycloheximide- or viomycin-producing strains were small enough for possible use as vectors.

From S. rochei producing lankacidin and lankamycin, 3 kinds of plasmids were isolated using the electrophoretic method, not by CsCl-EtBR centrifugation. Two linear and 1 circular plasmids were obtained from different variant strains and designated as pSLA 1,2, and 3 (Tab. 3).

pSLA 3, Circular, Transferable, and Pock-forming Plasmid

A circular plasmid 9, 2 kb was obtained from a S. rochei strain 7434 AN4 (3) with a high copy-number of 40 per chromosome. Its electrophoretic pattern and restriction map are presented in Fig. 1 and 2. There are 6 cleaving sites by SalI. There is no cut by EcoRI, HindIII, BglII, HpaI, SstI, XbaI, or XhoI. In a mixed culture of many spores of the 7434 AN 4 (2) strain with fewer spores of the 7434 AN 4 (3) strain, pocks were formed on the lawn of the former strain by lethal zygosis. The colony appearing in the clear zone

Tab. 3. Linear and circular plasmids isolated from S. rochei. LC: Lankacidin; LM: Lamkamycin.

strain	plasmid	antibiotics	resistance to LC
6642 GC1	pSLA 1 linear	LC, LM	+
7434 AN4(2)	pSLA 2 linear	LC, LM	+
7434 AN4(3)	pSLA 3 circular	LM	+
7343 LZ4(2,3)	pSLA 2 + 3	LC, LM	+



1. λ/HindIII
2. S. sp. 6642-GC_1 (pSLA1)
3. S. sp. 7434-AN_4 (2) (pSLA2)
4. S. sp. 7434-AN_4 (3) (pSLA3)

Fig. 1. Presence of 2 linear plasmids, pSLA 1 and pSLA 2, and circular transferable plasmid pSLA 3 in 3 strains of Streptomyces rochei.

was transferred to the pSLA 3 plasmid, giving rise to the strain 7434 LZ 4 (2,3) which contained both pSLA 2 and 3. The labeled pSLA 3 DNA had a weak homology with the left part of pSLA 2 linear plasmid DNA, as proven by the Southern hybridization technique.

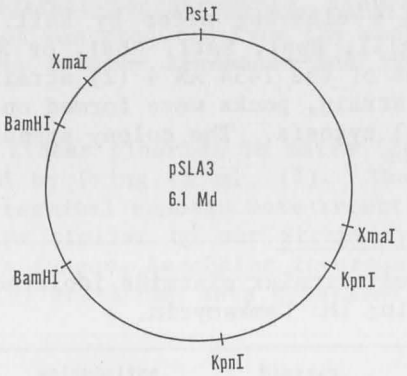


Fig. 2. Restriction map of pSLA 3.

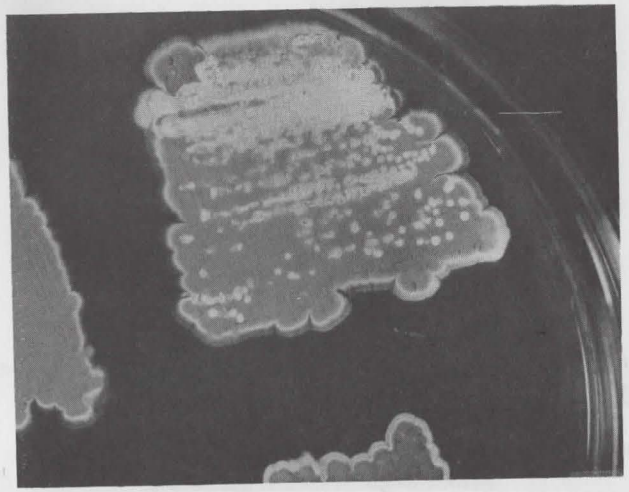


Fig. 3. Pock formation by the conjugative transfer of pSLA 3 plasmid from S. rochei 7434-AN4(3) to 7434-AN4(2).

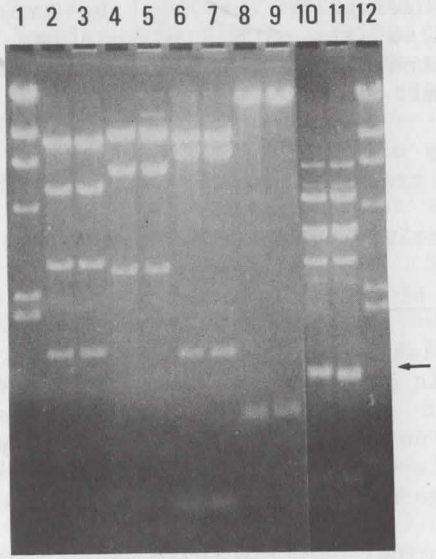


Fig. 4. Agarose gel electrophoresis of pSLA1 and pSLA2 treated with different restriction endonucleases. The arrow indicates the position of XmaI-E fragment of pSLA1. 1, 12: λ DNA treated with HindIII; 2, 3: BamHI; 4, 5: SalI: 6, 7: BglI; 8,9: EcoRI; 10, 11: XmaI; 2,4,6,8,10: pSLA1; 3,5,7,9,11: pSLA2.

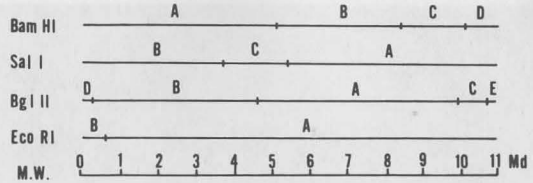


Fig. 5. Cleavage maps of pSLA2.

This transferable plasmid can be a good candidate as the vehicle in Streptomyces, for the following reasons:

a) Small molecular weight of 6.1 Md.

b) Unique cleaving site by PstI. Through a partial digestion technique, other cleaving sites can be used for cloning.

c) High copy number of 40 per chromosome which can result in the gene dosage effect for the greater expression of genes. Also, the pSLA 3 plasmid can easily be detected and isolated from a small quantity of cell cultures of even 0.5 ml.

d) Capability of "pock formation," which enables the isolation of a transferred clone with a high efficiency.

e) Transferability by mixed culture.

Restriction Pattern of Linear Plasmids pSLA 1 and pSLA 2 (3)

The 2 linear plasmids pSLA 1 and pSLA 2 DNA were isolated and their electrophoretic patterns were very similar (Fig. 4). The difference in size and number of the fragments generated by BamHI, SalI, and EcoRI was not detectable between the 2 plasmids. The only detectable difference was the XmaIE fragment where pSLA 1 was 60 bp longer. The cleavage map of the pSLA 2 DNA is presented in Fig. 5.

Association of Terminal Protein with the 5'-termini of Linear Plasmid DNA

Bacteriophages and animal viruses such as Bacillus subtilis ϕ 29 (10,11) and adenoviruses (12) contain linear DNAs. They have a unique structure at the ends of 5'-termini of their DNA, inverted repetitive sequences covalently attached to terminal proteins. The protein serves for the priming of DNA replication. We examined the possibility of the presence of such structures in pSLA 1 and pSLA 2. The attachment of protein to DNA can greatly reduce the electrophoretic mobility of DNA in the gel (10). We electrophoresed the

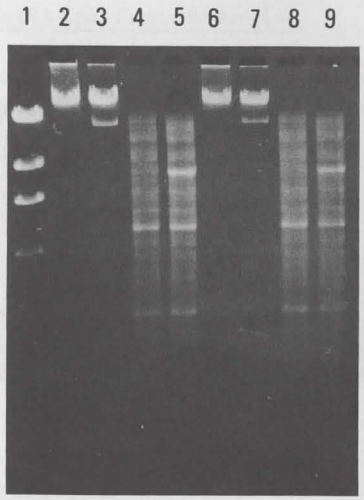


Fig. 6. Agarose gell electrophoresis of untreated or Pronase-treated pSLA1 and pSLA2. Cleared lysates prepared from pSLA1-harboring (2-5) or pSLA2-harboring (6-9) strains without Pronase treatment were subject to agarose gel electrophoresis after the following treatment. 2,6: No treatment; 3,7: treated with Pronase; 4,8: treated with BamHI; 5,9: treated with Pronase after BamHI digestion. 1: λ DNA treated with HindIII.

cleared lysates of S. rochei cells with or without the pronase treatment. The linear DNA bands were only detectable with the pronase treatment of the lysates. Furthermore, the same results were obtained in the case of BamHI-treated fragments of the linear DNAs; the 2 terminal fragments appeared only when they were treated with pronase (Fig. 6). These results indicate the firm binding of proteins to the 2 ends of the linear plasmid DNA.

We further examined the location of protein on pSLA 1 and pSLA 2. If a protein is associated with the end of the plasmid DNA, the DNA may be resistant to exonuclease, such as exonuclease III (3'-exonuclease) or λ-exonuclease (5'-exonuclease). The susceptibility of both plasmid DNAs to the 2 exonucleases was examined. As shown in Fig. 7, pSLA 2-protein complex was sensitive to exonuclease III, but resistant to λ-exonuclease. The same results were obtained in pSLA 1. These results strongly suggest that the 3' ends of these plasmid DNAs are free and the 5' ends are blocked by a protein covalently-bound.

The presence of this 5' end block is also assured by a 5' end-labeling experiment with polynucleotide kinase and [γ-^{32}P] ATP. The ^{32}P labeling of terminal DNA was doubled when the bound protein was removed by the alkali treatment of 0.1^{N} NaOH 30 Min (4).

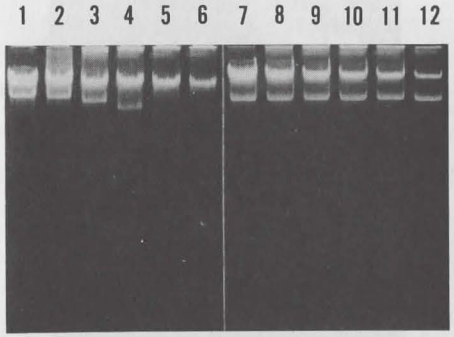


Fig. 7. Agarose gel electrophoresis of pSLA2 treated with exonucleases. Cleared lysates were treated with exonuclease III (1-6) or λ-exonuclease (7-12) for 0 min (1,7), 2 min (2,8), 5 min (3,9), 10 min (4,10), 15 min (5,11), or 40 min (6,12) at 37°C. After Pronase treatment, samples were subjected to agarose gel electrophoresis.

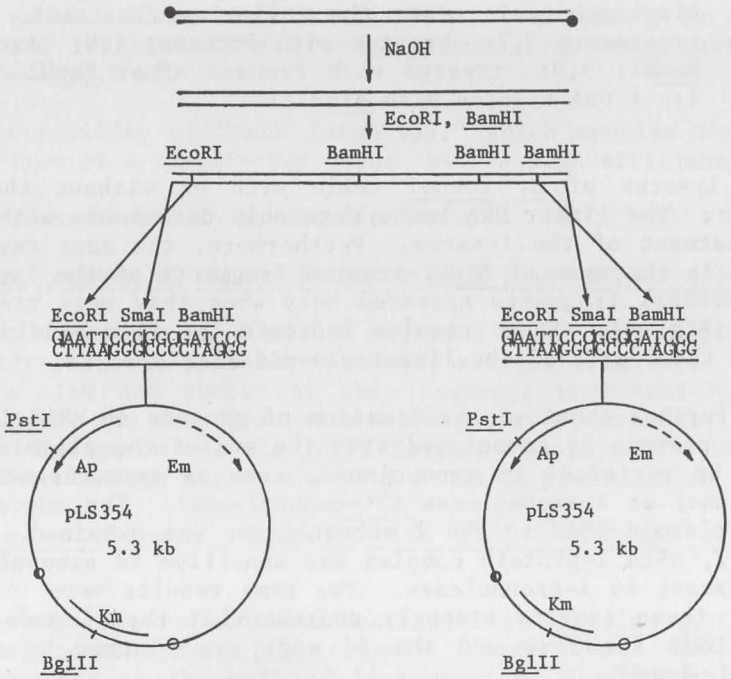


Fig. 8. Cloning of terminal fragments of pSLA2. The pSLA2 DNA was treated with NaOH in order to remove 5' end block (o).

Inverted Terminal Repetition Sequences (4)

The terminal fragments of the linear plasmid pSLA 2 were cloned in Escherichia coli cells using the plasmid pLS 354 (Fig. 8) The structure and the exact length of the inverted repetition were examined by determining the nucleotide sequence using the method of Maxam and Gilbert (13). Fig. 9 indicates the presence of an inverted terminal repetition of 614 bp. In the region beyond the 614 bp

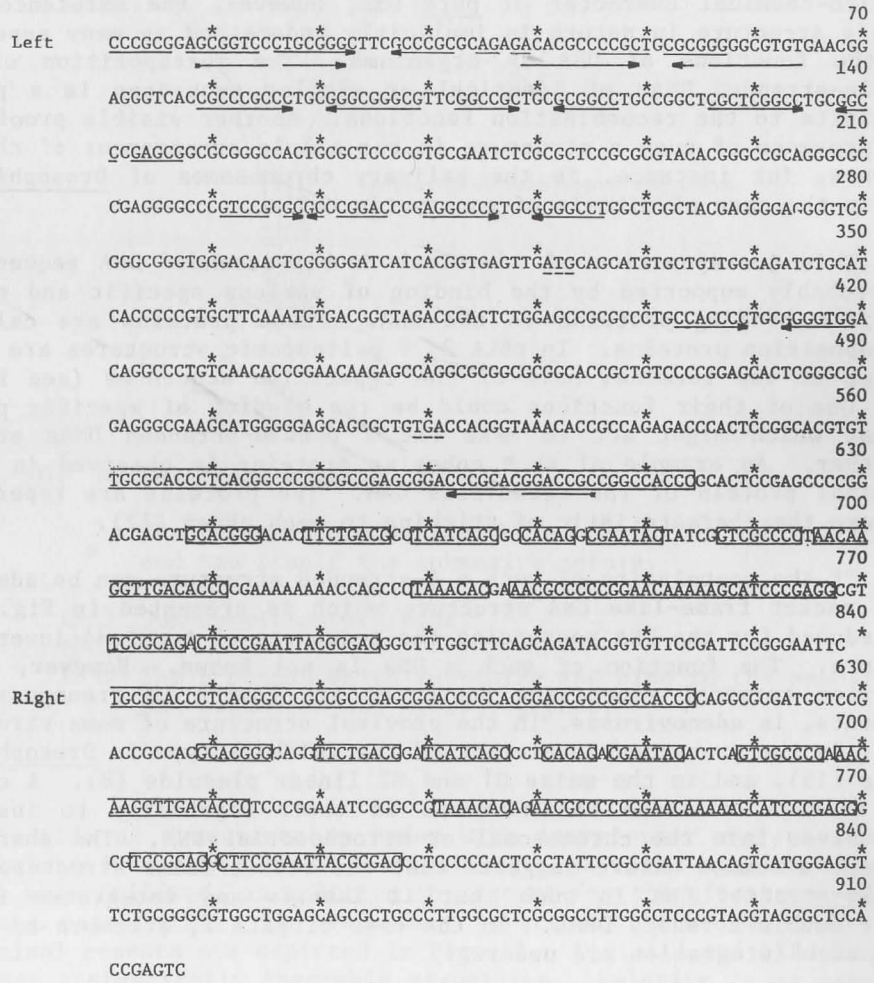


Fig. 9. Terminal nucleotide sequences of pSLA2. The inverted terminal repetition extends to nucleotide 614. The sequences homologous between the 2 ends are boxed. The palindromic sequences are indicated by the arrows. The initiation and termination codons of a possible coding sequence are indicated by the broken underlining.

of complete terminal homology, there are still 11 sites of interrupted homologous sequences which are indicated by boxes in Fig. 9. The total length of homologous sequences in the region from 615 to 800 bp from both ends reached 65%.

Juxtaposing 4 Strands of DNA and the Racket Frame Model

We postulate the presence of a juxtaposing structure of 4 strands of DNA. Such a structure cannot be considered from the physico-chemical character of pure DNA; however, the existence of such a structure in nature is implicitly understood in many aspects of the functions of DNA in organisms. The juxtaposition of 2 double-stranded DNAs of identical or similar sequences is a prerequisite to the recombination reactions. Another visible proof of the presence of such a structure is the allele arrangement of chromosomes, for instance, in the salivary chromosomes of Drosophila, and in the case of mitosis of eucaryotic cells.

This juxtaposition of identical double-stranded DNA sequences is probably supported by the binding of various specific and non-specific binding proteins to the DNA. These proteins are called juxtaposition proteins. In pSLA 2, 9 palindromic structures are detected at the terminal half of the repetition sequences (see Fig. 9). One of their functions could be the binding of specific proteins, which might act to make the 2 double-stranded DNAs stick together. An example of such cohesive proteins is observed in the terminal protein of the adenovirus DNA. The proteins are reported to have the characteristic of sticking to each other (12).

If the postulation of such a 4-stranded structure can be admitted, racket frame-like DNA structure which is presented in Fig. 10 is deduced for the DNA possessing the sequences of terminal inverted repeats. The function of such a DNA is not known. However, the inverted terminal repetition sequences are common in transposable elements, in adenoviruses, in the proviral structure of some viruses (14), in the transposable element of the foldback type in Drosophila cells (15), and in the maize S1 and S2 linear plasmids (8). A common feature of these DNA elements is their capability to insert themselves into the chromosomal or mitochondrial DNA. The sharing of such a common nature suggests that the racket-frame structure of double-stranded DNA is such that it inserts and integrates into other double-stranded DNAs. In the case of pSLA 2, attempts to detect such integration are underway.

Hairpin Structures at Termini of Inverted Repeats

From computer data analysis of the possible secondary structure of pSLA 2 DNA termini from 1 to 70 bp, several hairpin structures can be depicted on the basis of a low free-energy requirement, which can easily be formed, perhaps by the binding of proteins. This was accomplished by the analysis of matrices constructed to pair each

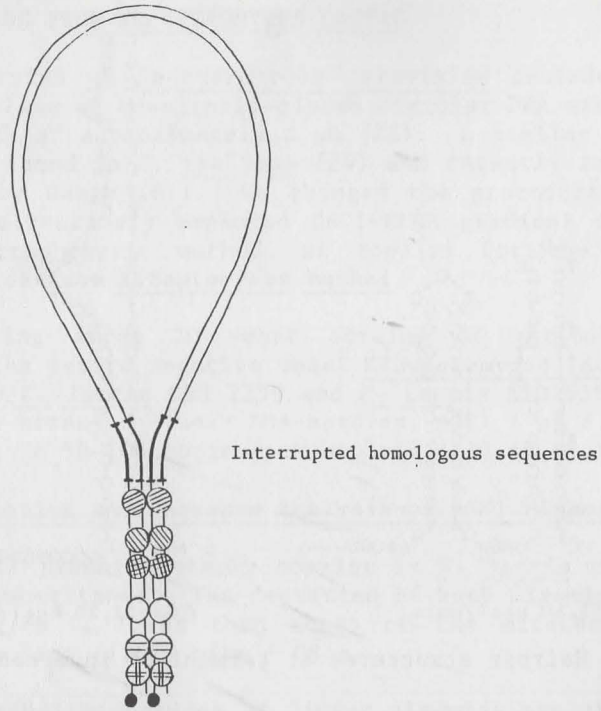


Fig. 10. Racket frame model.

● The terminal protein which initiates DNA replication and has itself the cohensive nature.

Juxtaposition proteins.

The proteins which recognize and bind to the specific DNA sequences and stock together.

nucleotide of a sequence in order with every other nucleotide. To calculate free energies of base pairing, the rules of Tinoco et al. (16) were followed, as modified to account for the effects of neighboring pairs (17). Two secondary structures formed from the 5'-terminal repeats are depicted in Fig. 11. Fig. 11A and 11B show the 2 most energetically favorable structures. Relative to an unpaired single DNA strand, the free energies of formation of these base paired structures are -52.1 Kcal/mole and -43.2 Kcal/mole, respectively. Given the number of nucleotides in the repeat sequences and the numbers of G-C pairs within the folded structures, 2 energies indicate a high degree of stability of the structures shown, and they could be expected to form spontaneously in single-stranded sequences at the termini.

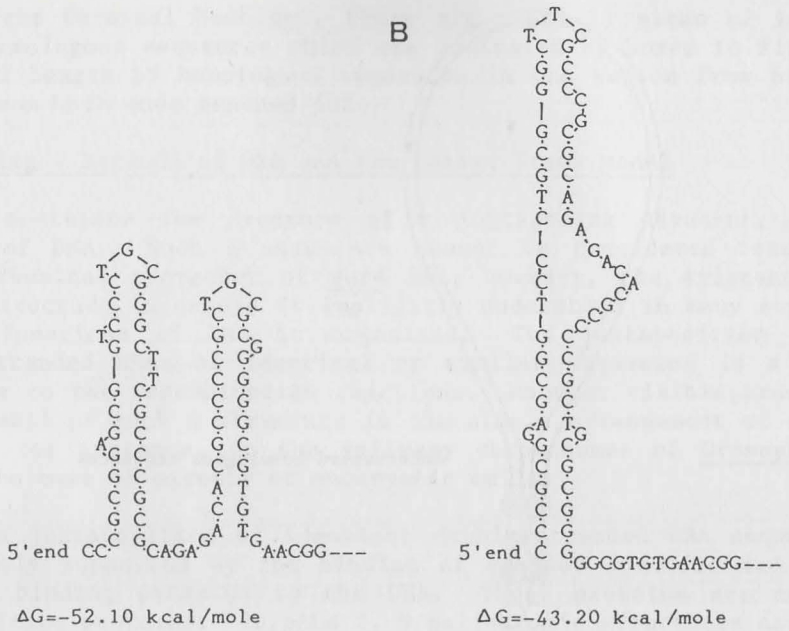


Fig. 11. Hairpin structures at termini of inverted repeats.

DISCUSSION

Various specific structures such as cohesive ends, direct or inverted repeats, or hairpins are usually present at the terminal region of linear DNA genomes (18). We have shown that the structure of the ends of pSLA 2 is similar to that of adenovirus DNA and Bacillus subtilis phage ϕ29 DNA: (a) these genomes have the inverted terminal repetitions and the terminal protein covalently linked to each 5' end; (b) the linkage between the genome and the terminal protein is alkali-labile, and 5' phosphate is released after alkali-treatment; (c) the ends of the genomes are blunt and contain free 3' OH. The terminal protein appears to function as a primer for DNA polymerase in adenovirus (12,19) and ϕ29 (20,21,22). This type of priming is called protein priming.

We have cloned intact termini of pSLA 2 after removal of the terminal protein with alkaline treatment. Using the same procedure, we have also succeeded in cloning the termini of pSLA 1 DNA, which showed an almost identical restriction pattern to that of pSLA 2, but had a small insertion or deletion of less than 100 bp in the nonterminal area. The nucleotide sequence of the cloned termini of pSLA 2 has been determined and an inverted terminal repetition of 614 bp long has been found. This is longer than that found in adenovirus, ϕ 29, and maize S1 or S2 plasmids (Lonsdale et al., 1981). The terminal sequence of pSLA 2 plasmid is highly homologous up to about 800.

LINEAR PLASMIDS FROM KLUYVEROMYCES LACTIS

Most strains of Saccharomyces cerevisiae contain the plasmid 2-μm DNA, a class of covalently-closed circular DNA with a monomeric contour length of approximately 2 μm (23). A similar type of plasmid has been found in S. italicus (24) and recently in other yeasts [see review by Gunge (6)]. We changed the procedure of detecting plasmids from routinely employed CsCl-EtBR gradient centrifugation to the electrophoretic method, as applied for the discovery of linear plasmids from Streptomyces rochei.

In looking among 70 yeast strains of various genera and species, in the petite negative yeast Kluyveromyces lactis IFO 1267, synonymous to K. lactis CBS 2359 and K. Lactis ATC 5356, we found 2 unique double-stranded linear DNA species, pGK1 1 of 8.9 kb and pGK1 2 of 13.4 kb, in 50-100 copies per haploid cell (5,6).

General Properties and Sequence Analysis of pGK1 Plasmid DNA

Both pGK1 plasmids stably coexist in K. lactis cells and show cytoplasmic inheritance. The densities of both plasmids are identical ($1{,}687$ g/cm^3), lower than those of the mitochondrial ($1{,}692$ g/cm^3) and nuclear ($1{,}699$ g/cm^3) DNAs.

The terminal structures of linear plasmids are of considerable interest, especially as to the DNA replication since DNA polymerase requires a primer to initiate replication. Several lines of evidence show that linear DNA molecules have unique terminal structures such as hairpin termini, palindromic termini, repetitive sequences, and protein association. Yeast chromosome ends (telomeres) are cross-linked and have repetitive sequences. Sequence analysis showed that pGK1 1 and pGK1 2 have repetitive unique ends. The termini consist of A-T rich inverted repeats of 202 bp in pGK11 and 184 bp in pGK1 2, as shown in Fig. 12 (F. Hishinuma, pers. comm.). These inverted terminal sequences were also reported by Sor et al. (25). There are several short dispersed repeats, either direct or inverted, but no internal palindromic sequence in the terminal area. Also, there is no obvious sequence homology between pGK1 1 and pGK1 2 in the area.

The total sequence of the shorter pGK1 1 plasmid which is responsible for the production of the killer toxin was found to be 8,876 bp with 5 open reading frames (H. Hishinuma et al., pers. comm.) (Fig. 13).

Protein Toxin Produced from pGK1 1 Plasmid

K. lactis cells harboring pGK1 plasmids secrete a toxic protein to kill sensitive strains over a wide range of yeasts, including Kluyveromyces, Saccharomyces, Candida, and Torulopsis, whereas the plasmid-containing cells are immune to the toxin. The purified toxin is composed of 2 subunits of proteins, one 27 kd and one over

```
pGK1 2

Left                                          AZuI
                                               ↓                    60
      AAAAGGTATA  TATATAAAAA  ATAAAGTTGG  GTTTTTAAGC  TAATAAAAGT  TGAAATCAGG
Right

                                                          Taq I
Left                                                Xho I  ↓↓
      TCAAAAAAAA  GCAACCCAAA  GTATGTTTTA  CGTGTTTTTT  TATGTTTAGG  TCATATCTCG
Right

      Rsa I          Hpa II  Hinf I                              180
Left  ↓              ↓       ↓                                   |
      AGTACGCCCT  TTTTCGCCGG  AGTCAATTAG  GTCATACTTT  TCTATATAAT  CCAAATCCCA
Right

                                                                240
Left       ATCAGT  TATAGTAGCA  TACAGTGTTA  AATGTATATT  TAATGTTACA  TAATAATTTA
      AAAA
Right      TCAATT  GAATGATTCT  TAATATGATT  TAATAGTTTA  TGATTATAAA  TGTCTTATAT
                     ↑                                                  ↑
                   Hinf I                                            Cla I


pGK1 1

                          Rsa I                          Rsa I  Mbo II
Left                       ↓                              ↓      ↓     60
      ACACATAACA  TAGGGGAGAG  TACTAAAAGT  GAGATTATTG  GAAGATTAGT  ACGTCTCCAT
Right

Left                                                              120
      TTTTTTCTGT  TTTTTTGTTT  TTATATATTA  GGTTATTTTT  TTTCAGTTTT  ATATCAACTC
Right

Left                                                              180
      TGTATAACAA  GTCTATTTTT  TTATATTTTA  AGTCTATTTT  ACACTTTTGA  CCTATAAGTC
Right

                                                                  240
Left                          ACTATAAT  ATATGAATTA  CATTATTAAT  TTAAAAATGG
      ATTTTATTAT  ACACATTTTC  CA
Right                         TTCTGTAG  ATTATTCATA  CTATCTATTT  TTTCAGTACA
                                                                  ↑
                                                                Rsa I
```

Fig. 12. Inverted terminal repetitive sequences of pGK1 1 and pGK1 2.

80 kd (26). The subunits are further characterized as containing another polypeptide of 24 kd (T. Fujimura, pers. comm.). The purified toxin protein blocks the growth of the sensitive cells of S. cerevisiae by inhibiting the adenylate cyclase activity in the membrane fraction (27) (Fig. 14). This is the first indication among the bacteriocin- type protein toxins of blockage of adenylate cyclase in the membrane. It is to be noted that the dsRNA encoded S. cerevisiae toxin is a simple protein of 11,740 d and distorts the ion transport of yeast membranes (28).

Genetic Analyses

Intergeneric transfer of both pGK1 1 and pGK1 2 into S. cerevisiae was achieved either by protoplast fusion (29) or by

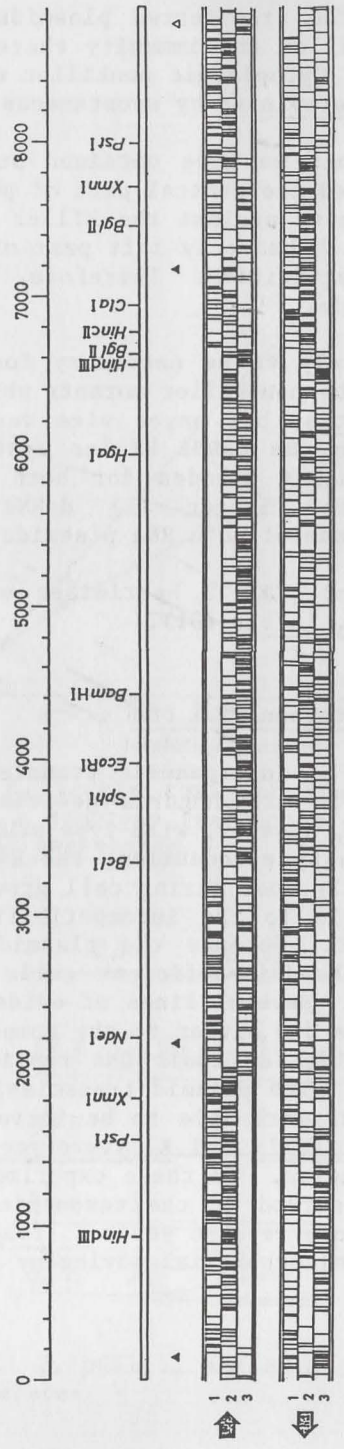


Fig. 13. Open reading frames present in pGK1 linear plasmid DNA.

transformation (30). The transferred plasmids exist in the cytoplasm and confer the killer and immunity characteristics on the S. cerevisiae host cells. Cytoplasmic nonkiller mutants were isolated from K. lactis and S. cerevisiae by spontaneous or induced mutation.

The deletion mutants we have obtained are shown in Fig. 15. The pGK1 1S which deleted the central part of pGK1 1 lost the killer activity, but the immunity against the killer protein was present. The other 2 mutants which had only left part of the plasmid, singly or doubled, lost both activities. Therefore, the immunity gene is located on the right side.

The pGK1 2 is likely to be necessary for the replication of both plasmids because the non-killer mutants which deleted only pKG1 1 was found in K. lactis, but never vice versa. Thus, the pGK1 killer system resembles the dsRNA killer system of S. cerevisiae where the smaller (M)dsRNA encodes for both killer and immunity phenotypes, whereas the larger (L) dsRNA is necessary for replication or maintenance of both RNA plasmids.

Neither pGK1 1 nor pGK1 2 hybridizes with the nuclear and mitochondrial DNAs of K. lactis (31).

Incompatibility with Mitochondrial DNA

In the experiment of intergeneric transfer, the pGK1 plasmids are stably maintained in mitochondria deficient (ρ^-) cells of S. cerevisiae (29,30). If, however, wild-type mtDNA (ρ^+) is introduced from a wild-type strain by cytoduction, the killer DNAs become unstable and are frequently lost during cell growth. This phenomenon appears to be ascribable to the incompatibility of pGK1 plasmids with the incoming mtDNA, because the plasmid stability could be restored in some mitochondria-deficient cells induced by ethidium bromide treatment (29). Several lines of evidence suggest that the incompatibility could be due either to the competition between mitochondria and the plasmids for their DNA replication, or to interference of mitochondria with plasmid transmission during cell division. The pGK1 plasmids were able to be introduced from K. lactis into Candida pseudotropicalis and Kluyveromyces fragilis by protoplast fusion or rare mating. In these experiments, the plasmid instability was again observed in the taxonomically distant Candida yeast, but not in the more related yeast K. fragilis. This suggests a relationship between mitochondrial phylogeny and incompatibility.

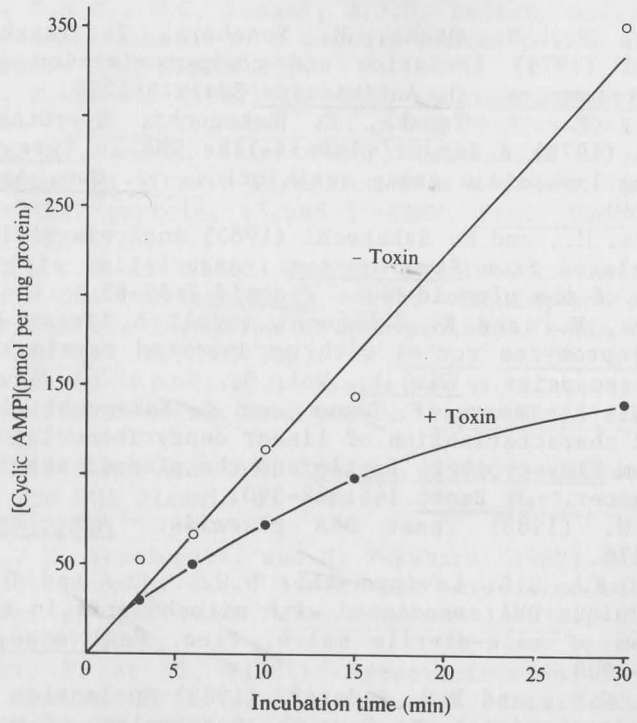


Fig. 14. Effect of K. lactis toxin on the activity of adenylate cyclase in a membrane fraction prepared from a sensitive strain of S. cerevisiae.

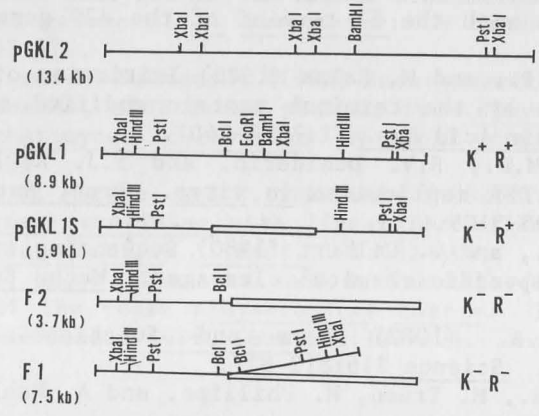


Fig. 15. Maps of pGK1 2, pGK1 1, and deletion mutants. The boxed areas are deleted.

REFERENCES

1. Hayakawa, T., N. Otake, H. Yonehara, T. Tanaka, and K. Sakaguchi (1979) Isolation and characterization of plasmids from Streptomyces. J. Antibiotics 32:1348-1350.
2. Hayakawa, T., T. Tanaka, K. Sakaguchi, N. Otake, and H. Yonehara (1979) A linear plasmid-like DNA in Streptomyces sp. producing lankacidin group antibiotics. J. Gen. Appl. Microb. 25:255-260.
3. Hirochika, H., and K. Sakaguchi (1982) Analysis of linear plasmids isolated from Streptomyces: Association of protein with the ends of the plasmid DNA. Plasmid 7:59-65.
4. Hirochika, H., and K. Sakaguchi (1984) A linear DNA plasmid from Streptomyces rochei with an inverted terminal repetition of 614 base pairs. EMBO J., Vol. 3, No. 4:761-766.
5. Gunge, N., A. Tamaru, F. Ozawa, and K. Sakaguchi (1981) Isolation and characterization of linear deoxyribonucleic acid plasmids from Kluyveromyces lactis and the plasmid associated killer character. J. Bact. 145:382-390.
6. Gunge, N. (1983) Yeast DNA plasmids. Ann. Rev. Microb. 37:253-276.
7. Pring, D.R., C.S. Levings III, W.W.L. Hu, and D.H. Timothy (1982) Unique DNA associated with mitochondria in the "S" type cytoplasm of male-sterile maize. Proc. Natl Acad. Sci., USA 74:2904-2908.
8. Levings, C.S., and R.R. Sederoff (1983) Nucleotide sequence of the S2 mitochondrial DNA from the S-cytoplasm of maize. Proc. Natl. Acad. Sci., USA 80:4055-4059.
9. Francou, F. (1981) Isolation and characterization of a linear DNA molecule in the fungus Ascobolus immersus. Mol. Gen. Genet. 184:440-444.
10. Ito, J. (1978) Bacteriophage ϕ29 terminal protein: Its association with the 5' termini of the ϕ29 genome. J. Virol. 28:895-904.
11. Mellado, R.P., and M. Salas (1983) Initiation of phage ϕ29 DNA replication by the terminal protein modified at the carboxyl end. Nucleic Acid Res. 11:7397-7407.
12. Chalberg, M.D., S.V. Desiderio, and T.J. Kelly, Jr. (1980) Adenovirus DNA replication in vitro. Proc. Natl. Acad. Sci., USA 77:5105-5109.
13. Maxam, A.M., and W. Gilbert (1980) Sequencing end-labelled DNA with base-specific chemical cleavages. Meth. Enzymol. 65:499-560.
14. Varmus, H.A. (1982) Form and functions of retroviral proviruses. Science 216:812-827.
15. Potter, S.S., M. Truen, M. Phillips, and A. Mahler (1980) Cell 20:639-647.
16. Tinoco, Jr., I., O.C. Uhlenbeck, and M.D. Levine (1971) Nature 230:362-367.
17. Tinoco, Jr., I. et al. (1973) Improved estimation of secondary structure in ribonucleic acid. Nature 246:40-41.

18. Kornberg, A. (1980) DNA replication (Freeman, San Francisco)
19. Rekosh, D.M.K., W.C. Russel, A.J.D. Bellet, and A.J. Robinson (1977) Identification of a protein linked to the ends of adenovirus DNA. Cell 11:283-295.
20. Watabe, K. et al. (1982) In vitro replication of bacteriophage ϕ29 DNA. Proc. Natl. Acad. Sci., USA 79:5245-5248.
21. Penalva, M., and M. Salas (1982) Initiation of phage ϕ29 DNA replication in vitro: Formation of a covalent complex between the terminal protein, p^3, and 5'-dAMP. Proc. Natl. Acad. Sci., USA 79:5522-5526.
22. Blanco, L., et al. (1983) Factors involved in the initiation of phage ϕ29 DNA replication in vitro: Requirement of the gene 2 product for the formation of the protein P^3-DNA complex. Nucleic Acid Res. 11:1309-1323.
23. Clark-Walker, G.D. (1972) Isolation of circular DNA from a mitochondrial fraction from yeast. Proc. Natl. Acad. Sci., USA 69:388-392.
24. Jazwinski, S.M., and G.M. Edelman (1979) Replication in vitro of the 2μm DNA plasmid of yeast. Proc. Natl. Acad. Sci., USA 76:1223-1227.
25. Sor, F., M. Wesolowski, and H. Fukuhara (1983) Inverted terminal repetitions of the 2 linear DNA associated with the killer character of the yeast Kluyveromyces lactis. Nucleic Acids Res. 11:5037-5044.
26. Sugisaki, Y. et al. (1984) Characterization of novel killer toxin encoded by a double stranded linear DNA plasmid of Kluyveromyces lactis. Eur. J. Biochem. (in press).
27. Sugisaki, Y., N. Gunge, K. Sakaguchi, M. Yamasaki, and G. Tamura (1983) Kluyveromyces lactis killer toxin inhibits adenylate cyclase of sensitive yeast cells. Nature 304:464-466.
28. Bostian, K.A., J.E. Hopper, D.T. Rogers, and D.J. Tipper (1980) Translational analysis of the killer-associated virus-like particle dsRNA genome of S. cerevisiae: M dsRNA encodes toxin. Cell 19:403-414.
29. Gunge, N., and K. Sakaguchi (1981) Intergeneric transfer of DNA killer plasmids, pKG1 1 and pKG1 2, from Kluyveromyces lactis into Saccharomyces cerevisiae by cell fusion. J. Bact. 147:155-160.
30. Gunge, N., K. Murata, and K. Sakaguchi (1982) Transformation of Saccharomyces cerevisiae with linear DNA killer plasmids from Kluyveromyces lactis. J. Bact. 151:462-464.
31. Wesolowski, M., P. Dumazert, and H. Fukuhara (1982) Killer DNA plasmids of the yeast Kluyveromyces lactis. II. Restriction endonuclease maps. Curr. Genet. 5:199-203.

CONJUGAL PLASMID TRANSFER IN BACILLUS THURINGIENSIS

John S. Chapman and Bruce C. Carlton

Department of Molecular and Population Genetics
University of Georgia
Athens, Georgia 30602

INTRODUCTION

Bacillus thuringiensis (BT) is a gram-positive, sporeforming bacterium distinguished by its ability to produce parasporal protein crystals. These proteins, collectively known as δ-endotoxins, are toxic to a number of lepidopteran insects of economic importance as well as to certain dipterans which are important vectors of human disease (7). Over 20 different varieties of B. thuringiensis are currently recognized on the basis of flagellar antigens (6) and immunological (19) and toxicity (8) properties of the parasporal crystals.

Strains of B. thuringiensis typically have complex arrays of extrachromosomal plasmids, in some instances up to 11 plasmids in a single strain (11). Genetic analysis of several parental strains and cured derivatives has demonstrated a correlation between specific plasmids and δ-endotoxin production (12), the implicated plasmid being a different size in different varieties. The discovery that toxin plasmids could be transmitted at high frequencies between crystal-producing (Cry^+) and acrystalliferous (Cry^-) strains during growth in mixed culture (13) confirmed the localization of genes for toxin production on the same specific plasmids implicated from curing studies (14,15).

Conjugal transfer of plasmids is not limited to Escherichia coli and other gram-negative bacteria. It is a well-documented occurrence in such gram-positive genera as Streptococcus (9), Clostridium (2), and Streptomyces (16), and there are indications that it may occur in Bacillus subtilis (18,20). Conjugal proficiency and cell clumping are induced in the Streptococcus faecalis system by exposure of donor cells to small peptides ("sex pheromones") produced by recipient cells (10). Several peptides are produced by recipient cells, each being specific for induction of transfer of a particular plasmid.

Preliminary experiments in our laboratory (13) had suggested that the natural mechanism for plasmid transfer among B. thuringiensis strains was due to a conjugation-like process. This work confirms and extends those findings using as a model system a 75-Mdal toxin-coding plasmid from strain HD2, a serotype 1 prototype strain of B. thuringiensis.

METHODS

Strains

The strains used in this study are listed in Tab. 1, together with their relevant phenotypes and plasmid complements. Strains were either obtained from J.M. González of our laboratory, or derived in this work. Strains resistant to 50 µg/ml nalidixic acid or 12 µg/ml rifampicin were generated by ethylmethanesulfonate mutagenesis (3) followed by selection on antibiotic-supplemented nutrient agar. Strain HD2 encodes the toxin genes on a transmissible 75-Mdal plasmid.

Media and Materials

Strains were maintained on slants of nutrient salts agar and routinely grown in nutrient broth. Nutrient salts medium consisted

Tab. 1. Strains used in these studies and their properties.

Strain[a]	Phenotype; plasmid complement[b]	Reference
HD2 B. thuringiensis var. thuringiensis; serotype 1	wild-type; 150, 75, 57, 54, 37, 32, 7.6, 7.2, 6.2, 5.2	González et al. (14)
HD2-13	crystal-positive; 75, 54, 32, 7.6, 7.2, 6.2, 5.2	González et al. (14)
HD2-62	strep^r, crystal-negative; 54, 32, 7.6, 7.2, 6.2, 5.2	González et al. (14)
HD2-106	nal^r, strep^r, crystal-negative; 54, 32, 7.6, 7.2, 6.2, 5.2	This work
HD2-107	rif^r, strep^r, crystal-negative; 54, 32, 7.6, 7.2, 6.2, 5.2	This work
HD2-108	nal^r, crystal-positive; 75, 54, 32, 7.6, 7.2, 6.2, 5.2	This work

[a] Strains HD2-13 and HD2-62 are derived from HD2 by curing spontaneously and with $NaDODSO_4$; the antibiotic resistance markers were generated via ethyl methane-sulfonate mutagenesis.

[b] Plasmid complement is indicated in megadaltons. Abbreviations: strep^r, streptomycin-resistant; nal^r, nalidixic acid-resistant; rif^r, rifampicin-resistant

of 8.0 g/l nutrient broth, 15.0 g/l Bacto-agar, 0.1 g/l $CaCl_2$, 10.0 mg/ml $MnCl_2 \cdot 4H_2O$, 1.0 ml/l 1.0 M $MgCl_2 \cdot 6H_2O$; SCG medium, used for growing cells for agarose gel electrophoresis was as previously described (12), as were the SP I and SP II media used for transformation (1).

Bacto-agar, nutrient broth, and casamino acids were obtained from Difco. Antibiotics and enzymes were purchased from Sigma.

Plasmid Transfer Conditions

The basic protocol for plasmid transfer was that of González et al. (14), modified for specific experiments. A Cry$^+$ streptomycin-sensitive donor strain and a Cry$^-$ streptomycin-resistant recipient were grown separately in nutrient broth in a shaker incubator for 14-20 hr at 30°C. These stationary phase cultures were diluted 1:50 into fresh nutrient broth and incubated with shaking for 3-4 hr. Matings were then performed in nutrient broth by mixing 10 µl/ml of donor and recipient cultures, usually in a final volume of 2.0 ml, for 8-20 hr. The recipient population was selected by streaking onto streptomycin-supplemented nutrient salts agar.

The DNase I experiments were performed as above with 2 modifications. First, mixed cultures received 40 µl/ml DNase I or an equal volume of distilled water, and second, matings were performed in SP II medium whenever the used medium was to be employed in a B. subtilis transformation experiment to monitor DNase I activity. Transformation was performed according to Anagnostopoulos and Spizizen (1). The transforming DNA was pBC16, a 3-Mdal plasmid coding for tetracycline resistance.

U-tube experiments were done in a commercially available apparatus purchased from Bellco Biological Glassware, Vineland, NJ. The final volume of the mating mix was 10 ml, with the donor and recipient strains being inoculated at a concentration of 10 µl/ml each. Filters with a 0.45 µ pore size (Millipore Corp.) were used to separate the chambers containing donor and recipient cultures.

In matings where the effects of inhibitors were studied, the inhibitor-sensitive member of the mating pair was inoculated at a 10-fold higher concentration than the inhibitor-resistant mating pair member and the matings performed for 20 hr. Streptomycin sulfate was used at a concentration of 100 µg/ml; nalidixic acid, at 50 µg/ml; and rifampicin, at 12 µg/ml. Mating cultures received 8 µl/ml of inhibitor solution or distilled water.

To determine the effects of initial cell density on mating, 1.0 ml and 100 µl aliquots of donor and recipient cultures were centrifuged for 1.0 min in a microcentrifuge (Model 235A, Fisher Scientific Co.) and resuspended to the original volume in fresh nutrient

broth. This wash was designed to eliminate media carryover effects during mating. Donor and recipient strains were inoculated into nutrient broth (final volume 10.0 ml) at initial concentrations of 10 µl/ml and 100 µl/ml each, respectively.

Conditioned media for use in mating experiments were prepared by growing mixed or axenic cultures for 8 hr, removal of the cells by centrifugation, and sterilization of the media by filtration. In some of these experiments, overnight cultures were treated by diluting them into media consisting of 50% conditioned media and 50% fresh nutrient broth. Donor and recipient inocula were centrifuged in a microcentrifuge, resuspended in fresh nutrient broth, and inoculated to the appropriate medium at 100 µl/ml each in a final volume of 10.0 ml. In some experiments the conditioned media were supplemented with 5X nutrient broth.

To determine donor mating capability, logarithmically growing cultures of donor and recipient cells were diluted 1:10 into distilled water and were vortexed vigorously. Cell counts were performed microscopically using a hemacytometer, and replicated 3 times. The cultures were then further diluted to obtain donor concentrations of less than 10 cells/10 µl and recipient concentrations of 50 cells/10 µl. Recipient cells were present at a higher density to avoid the possibility of a recipient conjugating with more than one donor cell. Actual donor and recipient cell numbers were determined by spotting 10 fractions of 10 µl each onto nutrient agar, incubating for 8-12 hr, and counting with the aid of a dissecting microscope. Parallel matings were performed using 10 µl inocula of the diluted donor and recipient cultures in a volume of 1.0 ml and incubating for 18-20 hr. The mating cultures were diluted and plated onto nutrient salts medium with and without streptomycin to determine final donor and recipient cell numbers. After 48 hr of incubation at 30°C, colonies on the plates were counted and the frequency of Cry^+ transconjugants determined.

Determination of Plasmid Transfer Frequencies, Cell Numbers, and Growth

At the termination of each mating experiment, samples were streaked to antibiotic-supplemented nutrient salts plates and incubated at 30°C for 48 hr. Frequencies of Cry^+ transconjugants were determined by microscopy of randomly selected colonies or by visual comparison of colony morphologies. Cry^+ colonies are whiter and more glossy than adjacent acrystalliferous colonies. The random screening method generally results in higher frequencies of Cry^+ transconjugants, perhaps due to an unconscious bias in selecting colonies.

Growth of mixed and axenic cultures was monitored in 25-ml side arm flasks by following changes in culture turbidity with a Klett-Summerson colorimeter fitted with a red (660 nm) filter. Cell numbers were determined by direct microscopic counting using a hemacytometer or by serial dilution and plating on appropriate media.

RESULTS

Nature of the Plasmid Transfer System

The plasmid transfer process displays a requirement for cell-cell contact, as shown by the results of U-tube experiments (Tab. 2). Separation of the 2 chambers of the U-tube with a 0.45 µ filter eliminated transfer of the 75-Mdal plasmid, while in U-tubes without filters approximately 50% of the streptomycin-resistant recipients (strain HD2-62) acquired the crystal-producing phenotype associated with the 75-Mdal plasmid.

Transfer of the 75-Mdal plasmid between crystal-producing and acrystalliferous strains of HD2 was insensitive to the action of 40 µg/ml DNase I in 20 hr matings (Tab. 2). DNase I in filtered, used media was still active after this period of time, as shown by its ability to eliminate transformation of Bacillus subtilis MI 112 by pBC16 to tetracycline-resistance.

Plasmid transfer experiments using mutagenized derivatives of strains HD2-13 and HD2-62 resistant to inhibitors of DNA, RNA, and protein synthesis (nalidixic acid, rifampicin, and streptomycin, respectively) were conducted to determine the possible roles of these

Tab. 2. Effects of DNase I and cell-cell contact on plasmid transfer.

Mating pair[a]	Condition	Frequency of cry^+ transconjugants	n[c]
HD2-13 x HD2-62	control	48.7 ± 1.32	6
HD2-13 x HD2-62	+DNase I	47.2 ± 3.9	4
HD2-13 x HD2-62	u-tube without filter	49.8 ± 2.9	4
HD2-13 x HD2-62	u-tube with filter	0	3

[a] HD2-13 is a crystal-producing, streptomycin sensitive derivative of HD2; HD2-62 is crystal-negative, streptomycin resistant derivative.

[b] Frequency was determined by microscopic examination of 30-50 colonies per determination and are presented as the means plus and minus the standard error of the mean.

[c] n = number of determinations.

processes in plasmid transfer. If the role of the donor strain is only to provide DNA (via cell lysis) for a transformation-like process, the appearance of Cry^+ phenotype in the inhibitor-resistant recipients should be insensitive to inhibition of macromolecular synthesis in the donor strain. In these experiments, the donor strains were initially present at a 10-fold higher concentration than the recipient strain to counter the lack of growth of the sensitive donor strains in the presence of the inhibitors. Table 3 indicates that nalidixic acid, rifampicin, and streptomycin eliminated transfer of the Cry^+ phenotype. Thus, it appears the donor must continue to synthesize DNA, RNA, and protein in order for transfer to occur. Reciprocal experiments using a nalidixic acid-resistant donor (line 8) or when both donor and recipient were resistant (line 10) indicate that it is necessary for the recipient to synthesize DNA (as expected if repliconation is to occur) and that elimination of transfer by nalidixic acid was due to the inhibitor-sensitive phenotype of the nalidixic acid-sensitive member of the mating pair. The insensitivity of plasmid transfer to DNase I, the requirement for cell-cell contact, and the necessity for DNA, RNA, and protein synthesis are all consistent with the hypothesis that transfer of the 75-Mdal plasmid occurs via conjugation.

Tab. 3. Effects of inhibitors of macromolecular synthesis on plasmid transfer.

Mating pair[a]	Condition	Frequency of cry^+ transconjugants	n[c]
HD2-13 x HD2-62	control	48.7 ± 1.32	6
HD2-13 x HD2-62	+ streptomycin	0	3
HD2-13 x HD2-106	control	33.0 ± 4.0	3
HD2-13 x HD2-106	+ nalidixic acid	0	3
HD2-13 x HD2-107	control	36.4 ± 2.2	3
HD2-13 x HD2-107	+ rifampicin	0	3
HD2-108 x HD2-62	control	39.0 ± 3.2	3
HD2-108 x HD2-62	+ nalidixic acid	0	3
HD2-108 x HD2-106	control	36.01 ± 1.8	3
HD2-108 x HD2-106	+ nalidixic acid	37.4 ± 2.2	3

[a] HD2-13 is $crystal^+$, streptomycin sensitive; HD2-62 is crystal-negative, streptomycin resistant; HD2-106 is crystal-negative, nalidixic acid resistant; HD2-107 is crystal-negative, rifampicin resistant; HD2-108 is crystal-positive, nalidixic acid resistant.

[b] Frequency was determined by microscopic examination of 30-50 colonies per determination and are presented as means plus and minus the standard error of the mean.

[c] n = number of determinations.

Characterization of the Conjugal System

The kinetics of the appearance of Cry^+ transconjugants parallels that of the logarithmic growth of a mixed population of donor and recipient cells. Occasional Cry^+ transconjugants could be found in the lawn of a sample streaked from broth after 2 hr of mixed growth. The frequencies of Cry^+ transconjugants became measurable after 3-4 hr of mixed growth and attained final frequencies of 30-40%.

Conjugal transfer of the 75-Mdal plasmid was independent of the initial cell density. The appearance and final frequency of transconjugants was identical in cultures receiving either the usual inocula ($2\text{-}4 \times 10^4$ cells/10µl of both donor and recipient) or 10 times the usual inocula (Fig. 1). Two implications may be drawn from these data: (i) that cell-cell contact is necessary but not sufficient for conjugation, and (ii) that one or both members of the mating pair must undergo a growth-related alteration in order to become conjugation proficient.

To lend credence to the idea that a growth-phase related change must take place for conjugation to occur, short matings (4 hr) were performed by mixing donor and recipient cells initially at different phases of the growth cycle. Strains HD2-13 and HD2-62 have identical growth rates in nutrient broth. Logarithmically growing cells show little or no lag when transferred to fresh nutrient broth, while stationary phase cells exhibit an extended lag of 2-3 hr before commencing logarithmic growth (Fig. 2). Thus, a culture established from logarithmically growing inocula can be considered to be in mid-log at the end of a 4-hr mating, while one derived from a stationary inoculum will be in early log phase after 4 hr.

When both the recipient and donor inocula were in log phase, transconjugants began to appear at 2 hr and their frequency continued to increase throughout the course of the experiment (Tab. 4). When both donor and recipient inocula were in stationary phase, no Cry^+ transconjugants were found after 4 hr. If the donor inoculum was in the stationary phase and the recipient inoculum was in log phase, again, no transconjugants were found after 4 hr. However, if the donor inoculum was in log phase and the recipient inoculum was in stationary phase, transconjugants began to appear after 3 hr. At this point the recipient cells were entering log phase and the donor cells were in late log phase. These results are interpreted as indicating that recipient cells can receive plasmids while in early log phase, while donor cells must be in mid- to late-log phase before they become conjugation proficient.

Formation of cell aggregates of 2 to several hundred cells (cell clumping) occurs during the growth of mixed or axenic cultures

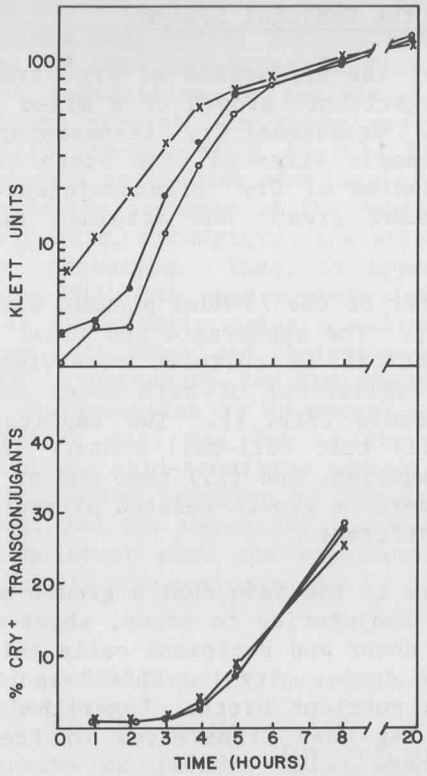


Fig. 1. Effect of initial cell density on growth of mixed cultures and transfer of crystal-producing phenotype. The top panel details the growth of mixed cultures of strains HD2-13 and HD2-62. Cells were centrifuged and resuspended in fresh nutrient broth before being used as inocula. Symbols: (○), 10 µl/ml inoculum of both donor and recipient; (×) 100 µl/ml inoculum of both donor and recipient; (●), uncentrifuged control, 10 µl/ml inoculum of donor and recipient. The lower panel describes the kinetics of appearance of crystal-producing transconjugants during growth of mixed cultures, as described in Panel A.

of B. thuringiensis. The fraction of the population in clumps increases as the culture progresses through logarithmic growth to stationary phase, although the clumping is not as extensive as that seen in the S. faecalis system. The data in Tab. 5 show that clumping in B. thuringiensis is nonspecific. Media used for the growth of any of the strains of B. thuringiensis examined will induce cell clumping in any of the other B. thuringiensis strains examined, including B. thuringiensis var. israelensis which does not conjugate with any of the strains listed in the table. Suspending cells in fresh nutrient broth produces no clumping.

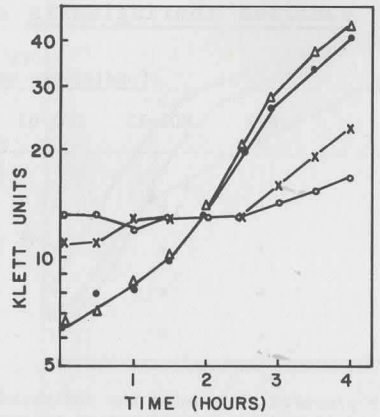


Fig. 2. Growth of strains HD2-13 and HD2-62 following inoculation from precultures at different growth stages. Symbols: (●), HD2-13, log inoculum; (○), HD2-13, stationary inoculum; (Δ), HD2-62, log inoculum; (×), HD2-62, stationary inoculum.

Pretreatment of either donor or recipient, or mixture of them with used media from donor, recipient, or mixed cultures prior to mating decreased transfer of the 75-Mdal plasmid to the recipient strain (Fig. 3). This effect is probably related to the increase in the length of the lag phase of the culture when transferred to the fresh broth used for the mating, most notably when both strains were treated.

Tab. 4. Effect of growth phase of donor and recipient inocula on plasmid transfer.

Growth Phase		Presence of cry$^+$ transconjugants[a]			
HD2-13	HD2-62	1 hr	2 hr	3 hr	4 hr
log	log	-	+	+	+
stationary	log	-	-	-	-
stationary	stationary	-	-	-	-
log	stationary	-	-	+/-	+

[a] Determined by microscopic examination of lawns of cells streaked onto nutrient salts medium supplemented with 100 µg/ml streptomycin sulfate. Several thousand cells were examined in each case; the experiment was repeated four times.

Tab. 5. Clumping of Bacillus thuringiensis cells by conditioned media.

Strain tested	Conditioned media from: HD2	HD2-13	HD2-62	HD567-61	Control[b]
HD2	+	+	+	+	-
HD2-13	+	+	+	+	-
HD2-62	+	+	+	+	-
HD567-61	+	+	+	+	-

[a] Conditioned media were prepared by growing the indicated strains in nutrient broth for eight hours; followed by centrifugation to remove the cells and filtration to resterilize the media.

[b] Control was fresh nutrient broth.

When cells grown in fresh broth were mated in media used in previous matings, or in media in which either donor or recipient strains alone had been grown, growth of the mixed culture proceeded at a lower rate although final cell densities were comparable to those obtained in fresh broth (Fig. 3). Conjugal plasmid transfer was severely inhibited in conditioned media from both mixed and axenic cultures of strain HD2. Frequencies of Cry^+ transconjugants in these experiments rarely exceeded 2%. Addition of nutrients (in the form of concentrated nutrient broth) did not alleviate the inhibition of conjugal transfer.

The addition of trypsin, pepsin, α-chymotrypsin, carboxypeptidase, protease K, or Staphylococcal V8 protease to mating mixtures had no effect on conjugation. These data, combined with those from the conditioned media experiments, would seem to preclude the possibility of involvement of peptides analogous to the sex pheromones of Streptococcus faecalis.

Mating Capability

If the ability to conjugate is limited to a specific subset of the donor cell population, dilution of the donor culture to obtain very small inocula sizes (i.e., <10 cells) would increase the chance that some donor inocula in parallel matings would not contain conjugation-proficient cells. Ideally, the donor inocula size would be one cell, however, the tendency of B. thuringiensis towards filamentous growth and cell clumping make it difficult to attain this ideal. The 3 experiments presented in Tab. 6 were selected from a larger number of experiments because they involved the smallest donor inocula (approximately 10 cells). The range of progeny:donor

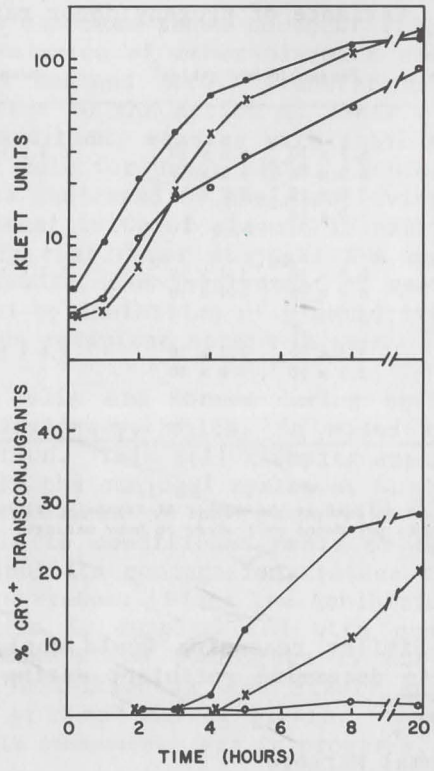


Fig. 3. Effect of conditioned media on growth of mixed cultures and transfer of crystal-producing phenotype. The top panel shows growth of mixed cultures of strains HD2-13 and HD2-62 under different conditions. Symbols: (●) derived from overnight cultures diluted into fresh broth, then mated in fresh broth; (×) derived from overnight cultures diluted into conditioned media, then mated in fresh broth; and (○) derived from overnight cultures diluted into fresh broth, then mated in conditioned broth. The lower panel describes the appearance of crystal-producing transconjugants under the same conditions described in Panel A.

ratios obtained in these experiments were similar to those obtained when using much larger inocula sizes (2-4 x 10^4 cells). In all experiments, progeny:donor ratios of less than one indicate that not all members of the donor population conjugated. Within experiments, the variance (a statistical measure of distribution about the mean) of progeny:donor ratios was very small compared to the mean, indicating the small segments of the population examined are not very different from the population as a whole. Thus, it seems likely that conjugal ability is not limited to a specific subset of the

Tab. 6. Variance of progeny:donor ratios.

Donor Inoculum[a]	Recipient Inoculum	Progeny:donor ratios[b]	Mean	Variance
11.5	40.2	6.7×10^{-2}, 6.6×10^{-2}, 8.3×10^{-2}, 1.1×10^{-2}, 7.2×10^{-2}, 8.2×10^{-2}, 5.5×10^{-2}	8.9×10^{-2}	5×10^{-4}
15	26	8.8×10^{-2}, 3.4×10^{-2}, 4.0×10^{-2}, 4.9×10^{-2}	5.2×10^{-2}	5.6×10^{-4}
9.6	40	3.1×10^{-1}, 2.2×10^{-1}, 2.1×10^{-1}, 3.0×10^{-1}	2.6×10^{-1}	8.2×10^{-3}

[a] Number of cells.

[b] Progeny:donor ratio is defined as the number of crystal positive transconjugants per donor cell after 20 hour matings.

donor population. Similar reasoning would apply to the recipient cells; experiments to determine recipient mating capability are in progress.

Transfer of Chromosomal Markers

In some conjugal systems (e.g., the F system of E. coli) mobilization of chromosomal genes by conduction occurs. In B. thuringiensis there are several possible instances of transmissible plasmids spontaneously integrating into the chromosome, and in some cases dissociating from the chromosome with and without size changes (Ref. 15 and J.M. González, pers. comm.). It is therefore reasonable to assume that chromosomal markers could be mobilized by the B. thuringiensis conjugal system. However, in numerous experiments designed to detect transfer of chromosomal markers at frequencies as low as 10^{-9}, no transmission of antibiotic-resistance markers (streptomycin, nalidixic acid, and rifampicin) or auxotrophic markers (histidine, tryptophan, and riboflavin) has yet been observed.

DISCUSSION

The data presented in this report lead to the conclusion that plasmid transfer in B. thuringiensis fits the classical definition of conjugation. It must be emphasized that this report has used, as a model system, the 75-Mdal toxin plasmid of strain HD2. However, transfer of many other plasmids in this strain and in other strains

of B. thuringiensis has been shown to occur (13,14) and we have n evidence that transmission of other plasmids occurs by a mechanis different than that defined here. Transfer of the 75-Mdal toxi plasmid is insensitive to the action of DNase I and requires cell cell contact. In addition, studies with inhibitors have indicate an active metabolic role for donor cells. Protein synthesis in th donor is required as indicated by the sensitivity of the process t streptomycin; the sensitivity of plasmid transfer to rifampicin an nalidixic acid imply that donor conjugal RNA and DNA synthesis i necessary (4). Likewise, the involvement of recipient conjugal DN synthesis is implied by inhibition of plasmid transfer by nalidixi acid when a sensitive recipient strain is used.

Aggregates of cells are formed during both mixed and axeni growth of B. thuringiensis, which, in mixed culture, presumably facilitates conjugation. This cell clumping appears to be nonspecific, however. Unlike the conjugal system of S. faecalis (10), there seems to be no involvement of pheromone-like peptides. In fact, preexposure of cells to conditioned media or mating the cells in conditioned media inhibits conjugation, rather than inducing it as in the Streptoccocus system. Since the inhibition also occurs when the conditioned media is supplemented with concentrated nutrient broth, it seems unlikely the inhibition is due simply to reduced growth. Thus, the inhibition is most likely due to a compound or class of compounds produced during growth. Further investigations of the nature of this phenomenon are in progress.

The frequency of crystal-producing transconjugants achieved in this mating system is slightly higher than those observed in other conjugation systems. The cointegrate plasmid pAD1::Tn 917 transfers to up to 10% of the recipient cells in the S. faecalis conjugal system (10), while in the gram-negative bacterium Pseudomonas stutzeri, plasmid markers transfer to up to 23% of the recipient population (21). The high frequencies seen in the B. thuringiensis conjugal system could be the result of many donor cells undergoing infrequent conjugations, or a few promiscuous cells conjugating many times. If the ability to conjugate is limited to a specific subset of the donor population, differences in progeny:donor ratios should be amplified in parallel matings where the donor inoculum size is very small (approximately 10 cells). Progeny:donor ratios of less than one indicate that not all donor cells conjugate; however, the variances observed within experiments is very small compared to the mean. This evidence implies, but does not unequivocally demonstrate, that conjugal proficiency is not limited to a subset of the donor population. Thus, while it seems that all donor cells may be able to conjugate, not all of them do. Factors affecting progeny numbers have been shown to include collision rate between donor and recipient cells, the abilities of both donor and recipient to mate, segregation of the plasmid in dividing recipient cells, and retransfer of the plasmid by recipients (5).

Although considerable progress has been made in the investigation of the B. thuringiensis conjugal system, much remains to be done. Mobilization of chromosomal markers has not yet been observed by us despite numerous attempts; work is in progress with different markers and different conditions to determine if this can be detected. The location of the genes responsible for conjugation is not certain, although the 75-Mdal toxin plasmid is an excellent candidate. Determination of the range of strains able to exchange plasmids conjugatively, and of which B. thuringiensis plasmids are mobilizable is another topic currently being investigated. A more complete understanding of the detailed mechanism of this genetic exchange system and the resultant ability to manipulate it will increase the importance of B. thuringiensis as a vehicle for the genetic engineering of biopesticides.

REFERENCES

1. Anagnostopoulos, C., and J. Spizizen (1961) Requirements for transformation in Bacillus subtilis. J. Bact. 81:741-746.
2. Brefort, G., M. Magot, H. Ionesco, and M. Sebald (1977) Characterization and transferability of Clostridium perfringens plasmids. Plasmid 1:52-66.
3. Carlton, B.C., and B.J. Brown (1981) Gene mutation. In Manual of Methods for General Bacteriology, P. Gerhardt, ed. Am. Soc. Microbiol., Washington, D.C.
4. Clark, A.J., and G.J. Warren (1979) Conjugal transmission of plasmids. An. Rev. Genet. 13:99-125.
5. Cullum, J., J.F. Collins, and P. Broda (1978) Factors affecting the kinetics of progeny formation with F'lac in Escherichia coli K12. Plasmid 1:536-544.
6. de Barjac, H. (1981) Identification of H-serotypes of Bacillus thuringiensis. In Microbial Control of Pests and Plant Diseases, 1970-1980, H.D. Burges, ed. Academic Press, New York, pp. 35-43.
7. Dulmage, H.T. (1979) Genetic manipulation of pathogens: Selection of different strains. In Genetics in Relation to Insect Management, M.A. Hoy and J.J. McKelvey, Jr., eds. Rockefeller Foundation, New York, pp. 116-127.
8. Dulmage, H.T. and cooperators. (1981) Insecticidal activity of isolates of Bacillus thuringiensis and their potential for pest control. In Microbial Control of Pests and Plant Diseases, 1970-1980, H.D. Burges, ed. Academic Press, New York, pp. 193-222.
9. Dunny, G.M., B.L. Brown, and D.B. Clewell (1978) Induced cell aggregation and mating in Streptococcus faecalis: Evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci., USA 75:3479-3483.
10. Dunny, G.M., R.A. Craig, R.L. Carron, and D.B. Clewell (1979) Plasmid transfer in Streptococcus faecalis: Production of multiple sex pheromones by recipients. Plasmid 2:454-465.

11. González, Jr., J.M., and B.C. Carlton (1980) Patterns of plasmid DNA in crystalliferous and acrystalliferous strains of Bacillus thuringiensis. Plasmid 3:92-98.
12. González, Jr., J.M., H.T. Dulmage, and B.C. Carlton (1981) Correlation between specific plasmids and δ-endotoxin production in Bacillus thuringiensis. Plasmid 5:351-365.
13. González, Jr., J.M., and B.C. Carlton (1982) Plasmid transfer in Bacillus thuringiensis. In Genetic Exchange: A Celebration and a New Generation, U.N. Streips, S.H. Goodgal, W.R. Guild and G.A. Wilson, eds. Dekker, New York. pp. 85-95.
14. González, Jr., J.M., B.J. Brown, and B.C. Carlton (1982) Transfer of Bacillus thuringiensis plasmids coding for δ-endotoxin among strains of B. thuringiensis and B. cereus. Proc. Natl. Acad. Sci., USA 79:6951-6955.
15. González, Jr., J.M., and B.C. Carlton (1984) A large transmissible plasmid is required for crystal toxin production in Bacillus thuringiensis variety israelensis. Plasmid 11:28-38.
16. Hopwood, D.A., and M.J. Merrick (1977) Genetics of antibiotic production. Bacteriol. Rev. 41:595-635.
17. Jacob, A.E., and S.J. Hobbs (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. J. Bact. 117:360-372.
18. Klier, A., C. Bourgouin, and G. Rapoport (1983) Mating between Bacillus subtilis and Bacillus thuringiensis and transfer of cloned crystal genes. Mol. Gen. Genet. 191:257-262.
19. Krywienczyk, J., and T.A. Angus (1967) A serological comparison of several crystalliferous insect pathogens. J. Invert Pathol. 9:126-128.
20. Orrego, C., M. Arnaud, and H.O. Halvorson (1978) Bacillus subtilis 168. Genetic transformation mediated by outgrowin spores: Necessity for cell contact. J. Bact. 134:973-981.
21. Stewart, G.J., C.A. Carlson, and J.L. Ingraham (1983) Evidenc for an active role of donor cells in natural transformation o Pseudomonas stutzeri. J. Bact. 156:30-35.

MECHANISMS ESSENTIAL FOR STABLE INHERITANCE OF MINI-F PLASMID

Sota Hiraga, Teru Ogura,* Hirotada Mori, and
Masafumi Tanaka

Institute for Virus Research
Kyoto University
Kyoto 606, Japan

INTRODUCTION

F plasmid of Escherichia coli is known to be stably maintained in dividing cells, despite of low copy number per cell. Mini-F plasmids, which were constructed in vitro from the 9.2 kb EcoRI-generated f5 fragment of the F plasmid, are also stably inherited, even though the copy number is still low (for review, see Ref. 1). We have sought to understand the molecular mechanisms involved in the stable maintenance of mini-F.

We have found that there are 2 different mechanisms for mini-F, besides the control mechanism of replication. The first mechanism presumably acts for equipartition of plasmid DNA molecules to daughter cells (2). This mechanism of partition depends on 2 trans-acting genes (sopA and sopB: sop stands for stability of plasmid) and cis-acting DNA site (sopC) of the plasmid (Fig. 1). The second mechanism acts to maintain stably plasmid by coupling host cell division to plasmid proliferation (3). This mechanism depends on 2 plasmid genes (ccdA and ccdB: ccd stands for coupled cell division). When replication of a plasmid carrying the ccdA and ccdB genes is prevented and the plasmid copy number decreases through subsequent cell divisions to a critical threshold, as few as 1 per cell, increase of colony formers is inhibited, and appearance of plasmid-free segregants is effectively prevented (Fig. 2). We have proposed a hypothesis that the ccdB gene product inhibits cell division via an unknown mechanism, and the ccdA gene product suppresses the ccdB function, except when the copy number decreases to the critical threshold (3).

* Department of Technology, National Institute of Health, Tokyo 141, Japan.

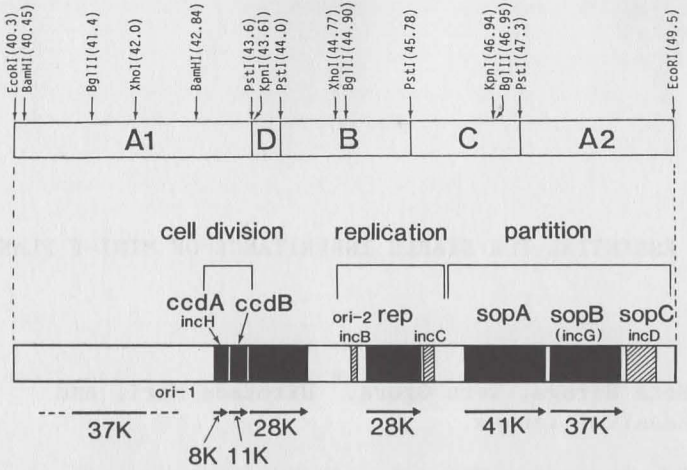


Fig. 1. Physical and functional map of EcoRI-generated f5 fragment of F plasmid. The numbers in parentheses denote kb coordinates on the F genome (1,3). The PstI segments are called A1, D, B, C, and A2 according to Murotsu et al. (4). The ori-1 and ori-2 denote replication origins of the mini-F plasmid (1). The rep codes for a protein essential for replication from ori-2 (5,6,7). The sop region essential for plasmid partitioning contains sopA, sopB, and sopC (2). The ccd region (coupled cell division) essential for coupling of host cell division to plasmid proliferation contains ccdA and ccdB (3). The incC (1), incB (1), incD (1,2,8), incG (2), and incH (Ref. 3, and see text) regions show incompatibilities. Filled regions on the map denote coding regions for proteins. Hatched regions denote direct repeats; 19 or 22 base-pair repeats in incB and incC (4,9,10), 43 base-pair repeats in incD (Takeyoshi Miki, pers. comm.). Arrows denote proteins (K, kilodalton) and their transcriptional direction (Ref. 1 and 11 and Takeyoshi Miki, pers. comm.).

MECHANISM OF PARTITION OF MINI-F PLASMID

Stabilization of oriC Plasmids by Joining with Mini-F Segments

We have described that F' plasmids carrying the replication origin (oriC) of the E. coli chromosome (12) or Salmonella thyphimurium (13) can replicate in Hfr strains of E. coli, in which F plasmid can not replicate. This suggests that a circular DNA molecule containing oriC can replicate as a plasmid in the cells using oriC. We have further isolated oriC plasmids from a transducing λ phage carrying oriC of E. coli by in vitro recombination techniques (14,15). We found that oriC plasmids are not stably maintained, and plasmid-free segregants appears at high frequencies in a nonselective medium. We assumed that the instability of oriC plasmids was due to the lack of function essential for equipartition.

To test this possibility, we constructed a composite plasmid (pXX11) consisting of an oriC plasmid and the mini-F plasmid (pSC138). The composite plasmid was stably maintained in Hfr strains, in which mini-F could not replicate. The oriC plasmid might be stabilized by the partition mechanism of mini-F (15).

We further constructed oriC plasmids carrying various segments of mini-F and examined the instability of these plasmids in F⁻ cells (recA1) to determine the minimal region necessary for stabilization of the oriC plasmid. These analyses indicate that the HaeII-SalI segment (46.4-49.4 kb in the F coordinate map) of mini-F is sufficient for the stabilization of the oriC plasmid (3). The stabilization of the oriC plasmid is achieved without any detectable increase in plasmid copy number per plasmid-carrying cell. This suggests that the HaeII-SalI segment is essential for the partition of the plasmid.

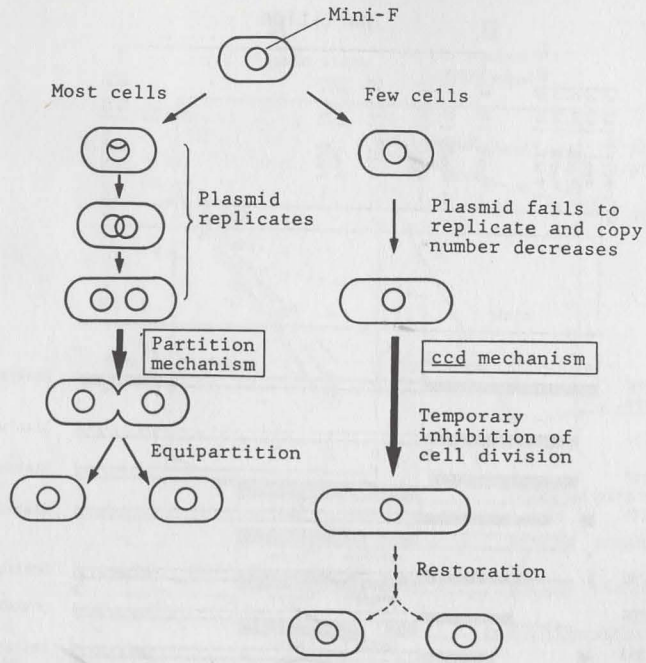


Fig. 2. Scheme showing mechanisms of stable inheritance of mini-F plasmid.

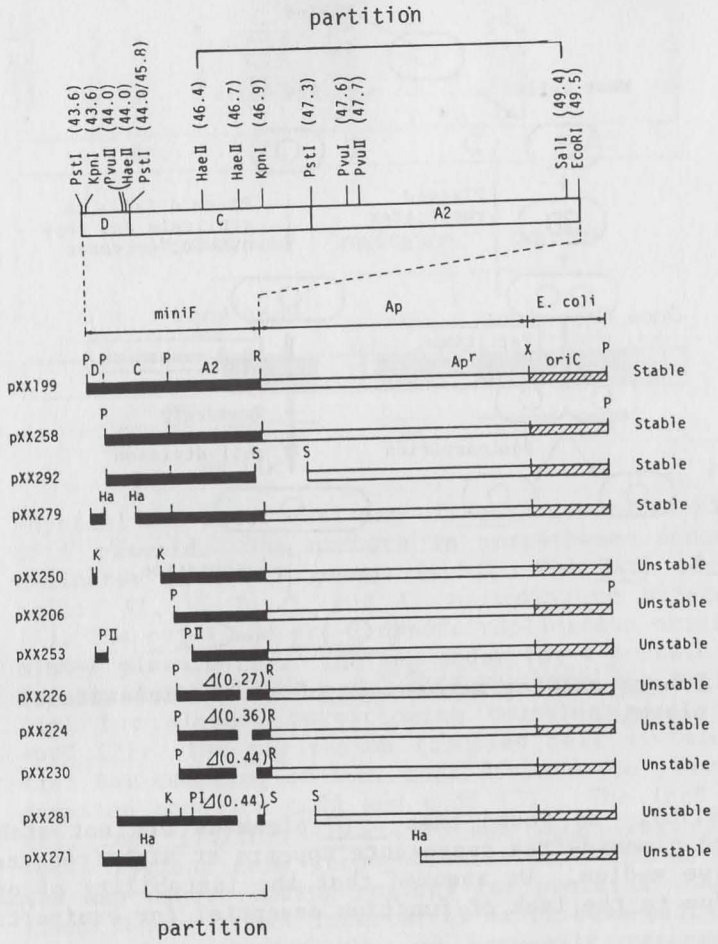


Fig. 3. Structure of deletion derivatives of an oriC plasmid carrying mini-F segments. The cleavage sites for EcoRI (R), PstI (P), and XhoI (X) are indicated. Only the sites for restriction endonucleases used to construct the deletion derivatives are shown on the map of each plasmid. S = SalI; Ha = HaeII; K = KpnI; PII = PvuII; PI = PvuI. The HaeII sites used to construct pXX279 are 44.0 kb and 46.4 kb on the F coordinate map, respectively. (Δ) A spontaneous deletion. (Number in parentheses = size of deletion in kb.)

Trans-Complementation Analyses of Functional Regions in the Partition Segment

To analyze the HaeII-SalI segment, we constructed 2 series of plasmids carrying various subsegments of the partition segment, us-

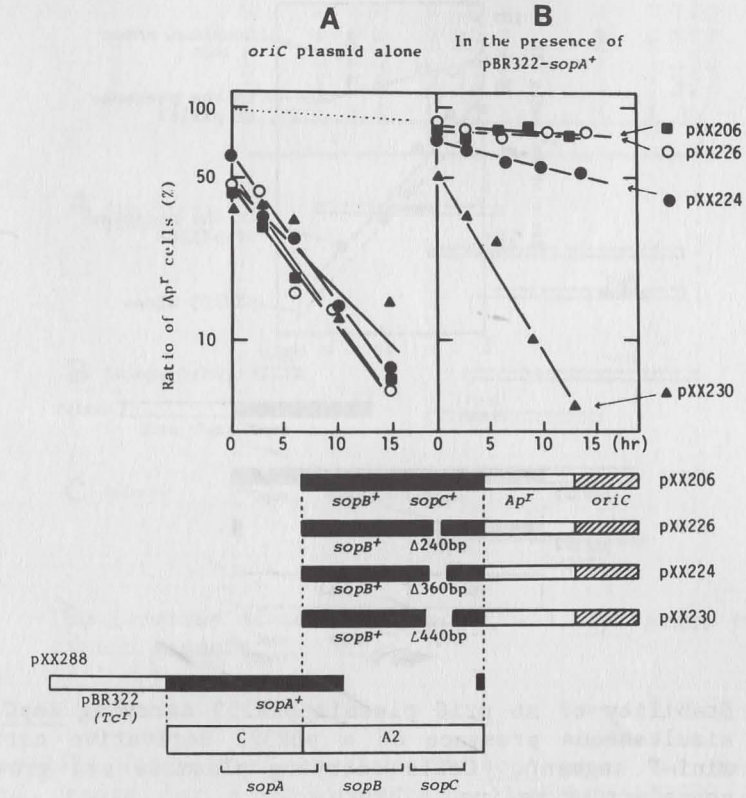


Fig. 4. Trans-complementation test for stability of oriC plasmids carrying a mini-F segment in the presence of a pBR322 derivative carrying a segment of mini-F. Samples were removed at intervals, diluted, and spread on L-agar plates. Colonies grown on the plates were tested for antibiotic-resistance. Dotted line in A shows the stability of pXX199 or pXX258 (see Fig. 3).

ing 2 vectors, i.e., an oriC plasmid and pBR322 (2). Stability of an oriC plasmid carrying a subsegment was measured in the presence or absence of a pBR322 derivative carrying another subsegment. These types of experiments were performed in various pairs of plasmids. Results from these complementation tests are shown in Fig. 4 and 5. Figure 4A shows stability of oriC plasmids carrying segments of mini-F in the absence of coexisting plasmids. All the oriC plasmids shown here are unstable. Figure 4B shows the stability of these oriC plasmids in the presence of pXX288, a pBR322 derivative carrying the PstI-HincII and HincII-EcoRI segments (47.3-47.9 kb and 49.4-49.5 kb, respectively). The oriC plasmid pXX206 was maintained stably in the presence of this pBR322 derivative. In contrast, the

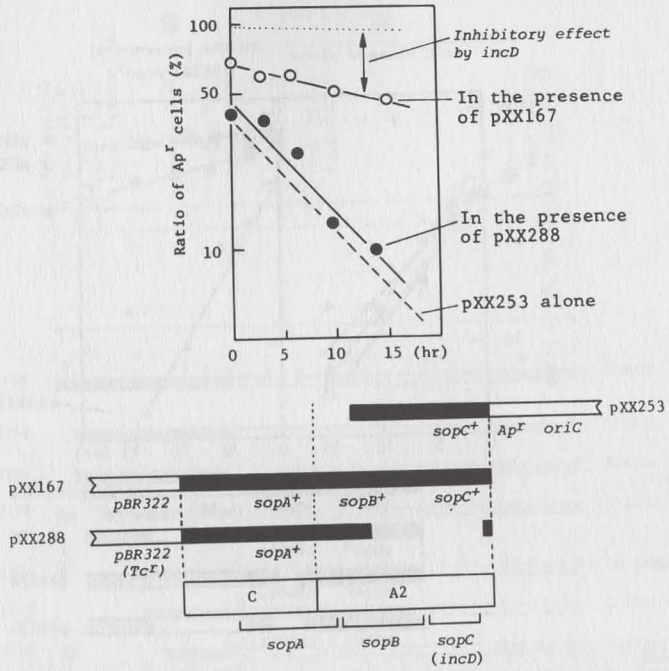


Fig. 5. Stability of an oriC plasmid pXX253 carrying sopC in the simultaneous presence of a pBR322 derivative carrying a mini-F segment. Cells carrying plasmids are grown in a nonselective medium, L broth.

pXX230 with a 440 base-pair (bp) deletion was still unstable. These results suggest that a trans-acting gene, designated sopA, essential for the stabilization is located in the mini-F segment carrying by pXX288. A cis-acting functional site, called sopC, which is located in the region deleted in oriC plasmid pXX230 is also involved in the stabilization.

The oriC plasmid pXX230 with a 360 bp deletion in the sopC region was partially stabilized by pXX288. With a 240 bp deletion, pXX226 was fully stabilized as pXX206. The activity of the sopC region, therefore, may be related to the size of the sopC segment.

As shown in Fig. 5, oriC plasmid pXX253 (see Fig. 3) is not stabilized in the presence of the pBR322 derivative carrying the sopA gene (pXX288), but can be partially stabilized by pXX167, which carries the entire segment essential for partition. This suggests that another trans-acting gene, termed sopB, exists between sopA and sopC. The oriC plasmid pXX253 is not fully stabilized by pXX167. This may be due to the incompatibility (incD) directed by the sopC region existing in both the plasmids (discussed later). These and further results suggest that sopA, sopB, and sopC are located in mini-F as shown in Fig. 6.

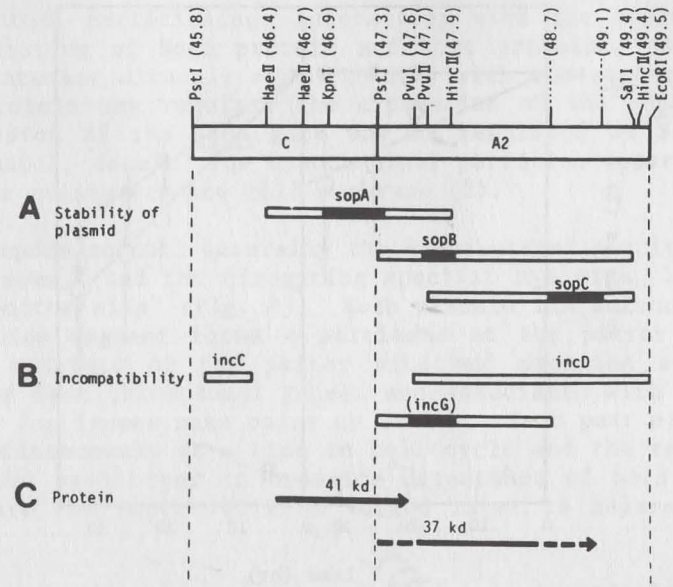


Fig. 6. The location of the sopA, sopB, and sopC genes in the partition segment.

A pBR322 derivative carrying the sopA gene, but neither sopB nor sopC, codes for a 41-kd protein. Another pBR322 derivative carrying the sopB gene, but neither sopA nor sopC, codes for a 37-kd protein. SopA and SopB proteins were recovered in cytoplasmic fraction and membrane fraction, respectively, in the presence of 10 mM $MgCl_2$ (K. Nagai et al., unpub. data). Austin and Wierzbicki (16) have isolated 2 amber mutants of mini-F which are not stably maintained. These mutant plasmids have a mutation in the genes coding for 41-kd and 37-kd proteins, respectively, consistent with our conclusion on sopA and sopB.

Isolation of Host Mutants Defective in Functions Essential for Partition of Mini-F

To investigate host chromosomal genes which are involved in the partition of mini-F, we isolated many host mutants in which an F' plasmid is unstable, and plasmid-free segregants appear at high frequencies under nonselective conditions. Then, we introduced a mini-F plasmid carrying the partition segment (pXX325, see Fig. 11) or that lacked the segment (pXX327, see Fig. 11) into these host mutants by transformation. Resulting transformants were analyzed for the stability of these mini-F plasmids in a nonselective medium. In one of the host mutants (hop-18, hop stands for host function of plasmid maintenance), the Sop$^+$ plasmid pXX325 was as unstable as the Sop$^-$ plasmid pXX327 (Fig. 7). This host mutant may be defective in a function essential for the partition of mini-F.

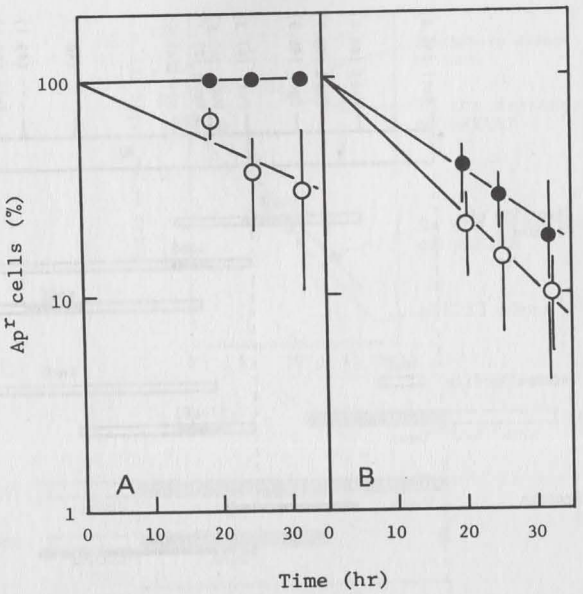


Fig. 7. Stability of mini-F plasmids in a host mutant (hop-18) defective in the essential function for partition of mini-F. Cells carrying a mini-F plasmid were spread on nonselective plates and incubated at 37°C for various times, indicated. To test for percent of plasmid-free segregants to total cells in each colony grown on the plates, colonies were independently removed, suspended in saline, diluted, and spread on nonselective plates. Colonies grown on the plates were removed with toothpicks and tested for ampicillin-resistance. Average ratio of ampicillin- resistant colonies to total colonies is shown in the figure. A vertical line at each circle denotes confidence limits ($\alpha = 0.05$). A, parental host (KZ344). B, hop-18 mutant. Solid circle, pXX325, a mini-F plasmid carrying the partition segment. Open circle, pXX327, mini-F plasmid lacking the partition segment. (see Fig. 11). Doubling time of cells was 50-55 min in all the strains.

In other host mutants tested, the Sop+ plasmid pXX325 was unstable at various levels, but the Sop⁻ plasmid pXX327 was extremely less stable, more than 10-fold, than pXX325. We, therefore, judged that this type of mutants was partially defective in the host function essential for the replication of mini-F.

Hypothetical Apparatus of Partition

We have described a model for the partition mechanism of mini-F plasmid (2). The sopC region acts as a specific DNA site necessary

for the actual partitioning, interacting with the cellular components consisting of SopB protein and host proteins. SopA protein may also interact directly or indirectly with the sopC region, and/or SopA protein may regulate the expression of the sopB gene (2). The expression of the sopA gene may be regulated by SopB protein (17; our unpub. data). The hypothetical partition apparatus is assumed to be related to the cell membrane (2).

We propose to call generally the hypothetical partition apparatus "partisome," and the cis-acting specific DNA site, like sopC of mini-F, "partor site" (Fig. 8). Each plasmid DNA molecule carrying the partition segment forms a partisome at the partor site. The partisome consists of the partor site and proteins specified by plasmid and host chromosomal genes, and associates with cytoplasmic membrane. Partisomes make pairs at random. Each pair of partisomes splits simultaneously at a time in cell cycle and the released partisomes move each other to opposite directions of both sides of a plane, where the septum will be formed later to separate daughter cells.

In the partisome model, when 2 kinds of plasmids having homologous partition mechanisms, but heterologous replication mechanisms, exist in a cell, a weak incompatibility between both plasmids should be observed, because random assortment of DNA molecules of the plasmids to daughter cells results from the partition. IncD phenotype directed by the sopC region of mini-F, therefore, may be due to the random assortment of plasmid molecules. This model is consistent with the speculation that random assortment in equipartition results in a weak incompatibility (18,19).

MECHANISM OF COUPLING OF HOST CELL DIVISION TO PLASMID PROLIFERATION

Novel Mechanism of Plasmid Maintenance

We have also described an additional novel mechanism which acts to stably maintain a plasmid (3). This mechanism is specified by 2 plasmid genes, ccdA and ccdB, which are located within the HpaI-PstI segment (42.9-43.6 kb; ccd region) of mini-F (see Fig. 1). A plasmid (e.g., oriC plasmid) carrying this segment is more stably maintained than that lacking the segment. An oriC plasmid carrying both the ccd segment and the partition segment of mini-F is extremely stably maintained as the parental mini-F pSC138.

When temperature-sensitive polA mutant cells (km1213), which harbor a pBR322 derivative carrying the ccd segment, pXX306 (see Fig. 10), were grown at 30°C in a nonselective medium and transferred to 42°C, colony formers (viable cells) continued to increase exponentially for 3-3.5 hr (3.5-4 generations) after transfer

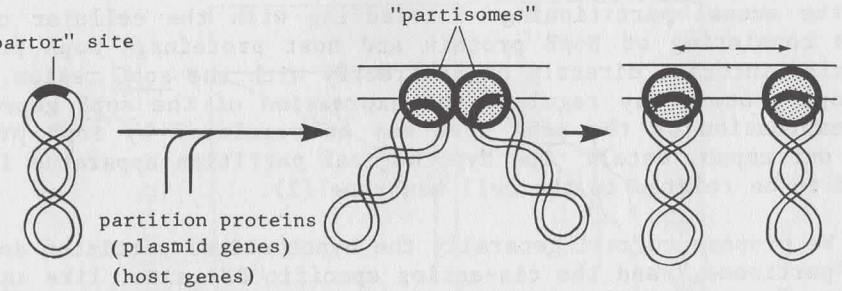


Fig. 8. Hypothetical apparatus for partition of plasmid in prokaryotes: "Partisome" model.

to 42°C, and then stopped increasing (Fig. 9B). Turbidity increased at least for 6-7 hr and filamentous cells with many nucleoids were observed. Plasmid-free segregants appeared only infrequently after 7 hr incubation at 42°C. In contrast, the inhibition of cell division was not observed in a strain carrying pBR322, and segregants began to appear at high frequencies after about 3 hr (Fig. 9A). It

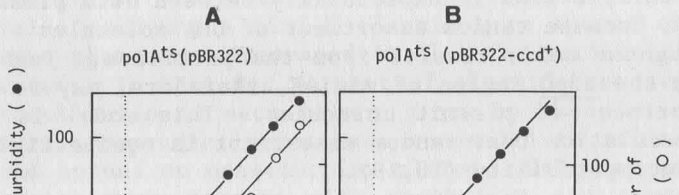


Fig. 9. Inhibition of host cell division in polA^{ts} cells which harbor a pBR322 derivative carrying the ccd segment, pXX306. Cells (polA^{ts}) carrying a plasmid grown at 30°C were transferred to 42°C, and then turbidity of the culture, colony formers and ratio of plasmid-carrying cells to total cells were measured at intervals. (A) polA^{ts} cells harboring pBR322. (B) polA^{ts} cells which harbors a pBR322 derivative carrying the ccd segment of mini-F, pXX306.

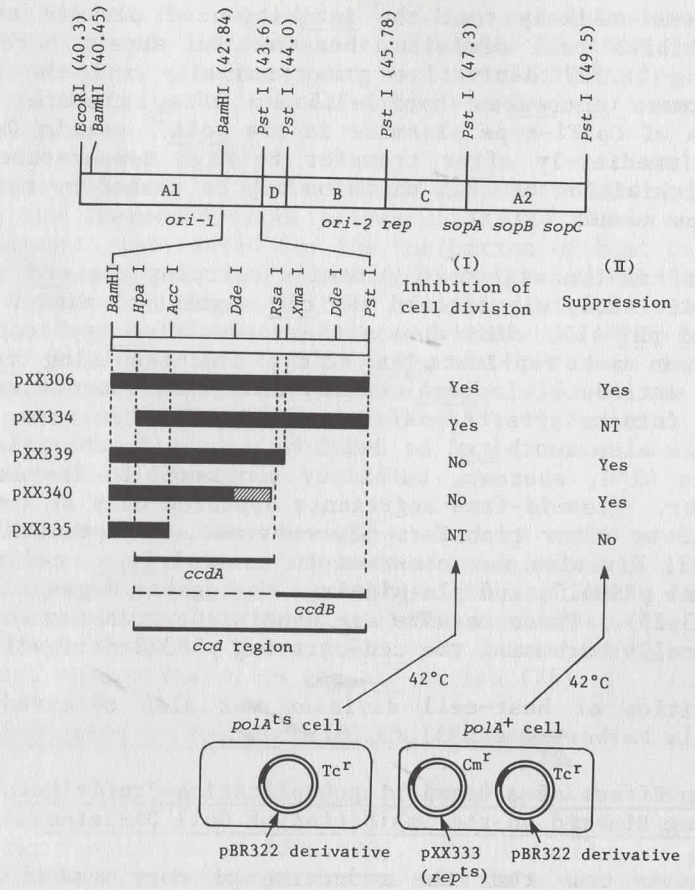


Fig. 10. Properties of pBR322 derivatives carrying mini-F segments. Column I, inhibition of cell division in polA^{ts} cells (km1213) carrying a pBR322 derivative carrying the indicated segment of mini-F (solid bar) after transfer to 42°C. Column II, suppression effect of the indicated plasmid on the inhibition of cell division which is caused at 42°C by a thermosensitive plasmid carrying the ccd region (PXX333) (see text). KH802 cells (polA^{+}) harboring both the pXX333 plasmid and the indicated pBR322 derivative were transferred to 42°C, and samples were removed and tested for colony formers. NT = not tested.

should be noted that the time when colony formers stop increasing in cells harboring the ccd-carrying pBR322 derivative nearly coincides with the time when plasmid-free segregants begin to appear in control pBR322-harboring cells. DNA synthesis of the cells harboring the ccd-carrying pBR322 derivative was not inhibited at least for 6 hr (3).

It seems unlikely that the inhibition of plasmid replication per se inhibits cell division, because the strain harboring the ccd-carrying pBR322 derivative grows normally and the number of colony formers increases for 3-3.5 hr after transfer to 42°C. Replication of ColE1-type plasmids in the polA^{ts} strain (km1213) is inhibited immediately after transfer to high temperature. Therefore, the inhibition of cell division may be caused by reduction of plasmid copy number (3).

To confirm the effect of plasmids carrying the ccd segment on host cell division, we recloned the ccd segment of mini-F on a vector plasmid pHSG415, which has a thermosensitive replication mutation and can not replicate at 42°C. The resulting ccd-plasmid pXX333 was introduced in recA$^+$ cells, and transformants were tested for colony formers after transfer to 42°C. In this case, host cell division was also inhibited at 2-2.5 hr (about 3 generations) after transfer to 42°C, whereas, turbidity continued to increase for at least 4.5 hr. Plasmid-free segregants appeared only at low frequencies at 4.5 hr after transfer. By contrast, no detectable inhibition of cell division was observed in control recA$^+$ cells carrying the parental pHSG415, and plasmid-free segregants began to appear at 2-2.5 hr (3,20). These results are consistent with the above result in polA^{ts} cells harboring the ccd-carrying pBR322 derivative.

Inhibition of host-cell division was also observed in recA1 mutant cells harboring pXX333 (3,20).

Suppression Effect of a Coexisting Replication-Proficient ccd-carrying Plasmid on the Inhibition of Cell Division

If it is true that the reduction of copy number of a ccd-carrying plasmid triggers a signal for inhibition of cell division, it could be expected that the inhibition is suppressed by the presence of replication-proficient plasmid carrying the homologous ccd segment. Accordingly, we introduced mini-F (pSC138) into the polA--ts strain which harbors the ccd-carrying pBR322 derivative of mini-F, and examined cell division after transfer to 42°C. As expected, cell division was no longer inhibited.

Similar suppression of the inhibition of cell division was observed in a polA$^+$ strain which harbored both the ccd-carrying thermosensitive plasmid pXX333 and the ccd-carrying pBR322 derivative. The inhibition of cell division caused by reduction of copy number of the ccd-carrying plasmid is thus suppressed by the replication-proficient plasmid carrying the homologous ccd segment.

Two Genes Located in the ccd Segment

To analyze the genes located within the ccd region, we constructed pBR322 derivatives carrying various subsegments of the ccd region, introduced them into polA^{ts} cells, and tested for the inhibition of host cell division after transfer to 42°C. As shown in Fig. 10 (column I), the functional region sufficient for the inhibition of cell division is located within the HpaI-PstI segment (42.9-43.6 kb) (3).

We introduced 1 of these pBR322 derivatives into polA$^+$ cells harboring the thermosensitive plasmid, pXX333, which carried whole the ccd segment, and tested for the inhibition of host cell division at 42°C. Results shown in Fig. 10 (column II) indicate that the suppression function on the inhibition of cell division is specified by a gene, named ccdA, which is located within the HpaI-RsaI segment (42.9-43.35 kb). Another gene, ccdB, which causes inhibition of cell division, may be located at the right side of the ccdA gene. The ccdA gene product suppresses the inhibitory function of the ccdB gene, except when the copy number of ccd-plasmid reduces to the critical threshold (3).

Similar phenomena have been described by other groups (21-24). Two proteins coded by the ccd segment were detected (21,22). A protein corresponding to CcdB protein inhibits cell division and another corresponding to CcdA protein suppresses the inhibitory function of the former. There are 2 open frames coding for 8 and 11 kd proteins, respectively, in the ccd region (24).

Prophage Induction by Functions of the ccd Genes

We found that weak induction of prophage λ was observed, besides inhibition of cell division, in a recA$^+$ λ-lysogen harboring pXX333 after incubation at 42°C (20). The induction of prophage in the pXX333-harboring λ-lysogen occurs only in a few percent of cells in the culture.

Both the λ induction and the inhibition of cell division caused by blocking of replication of pXX333 were suppressed in the presence of a replication-proficient pBR322 derivative carrying the ccd gene. The λ induction, but not the inhibition of cell division, depends on the recA$^+$ gene function. The signal caused by the ccdB gene function may inhibit cell division via an unknown recA-independent pathway. The weak induction of prophage may be due to the side effect of the ccdB function via a recA-dependent pathway.

Weak induction of synthesis of RecA protein was observed in the recA$^+$ strain harboring pXX333 after incubation for 3 hr at 42°C, but not in the recA1 mutant harboring this plasmid (20). Karoui et al (22) have described induction of prophage λ and SfiA protein synthesis caused by inactivation of the ccdA gene function of mini- F. Our recent data (A. Jaffe and S. Hiraga, in preparation) indicate that the inhibition of cell division by the ccd function is independent of sfiA and sfiC (25) functions.

It is also unknown what is the molecular mechanism which monitors directly or indirectly the decreased copy number to one copy of ccd-carrying plasmid. One possible explanation is that the rate of synthesis of CcdA protein depends on the copy number of the plasmid and, when CcdA protein is reduced to a critical concentration in the cell, the inhibitory function of CcdB protein appears and cell division is inhibited via a recA-independent pathway. If only oligomer of CcdA protein, but not monomer, had the suppression function against the inhibitory function of CcdB protein, the dependency of this phenomenon on the concentration of CcdA protein could be very clear, because, for example, the concentration of the dimer is in proportion to the 2 power of that of the monomer.

We have proposed, in addition, a hypothesis (20) that the ccd mechanism of F plasmid contributes to indirect induction (26) of prophage λ caused by an F plasmid transferred into a λ lysogen via conjugation.

STABILITY OF MINI-F PLASMID

Evidence of the Two Mechanisms Acting for Stable Inheritance of Mini-F

We have demonstrated that the partition mechanism and the ccd mechanism of mini-F act actually for stable inheritance of mini-F plasmid (2,3). We constructed deletion mutants of mini-F pSC138 and tested their stability. As shown in Fig. 12A, pXX9 and pXX317, which have both the Sop^+ and Ccd^+ functions, are stably maintained as the parental plasmid pSC138. By contrast, pXX318 and pXX344, which have the Ccd^+ function but not the Sop^+ function, are unstable (Fig. 12B). The Sop^+ function, therefore, acts for the stable maintenance of mini-F. Plasmid pXX327, which has neither the Sop^+ function nor the Ccd^+ function, is less stable than pXX318 and pXX344 (Fig. 12C). Therefore, the Ccd^+ function acts for stable inheritance of mini-F plasmid. Resulting from impaired equipartition of the Sop^- plasmid, pXX318 or pXX344, cells which have only 1 copy of the plasmid per cell may appear at a high frequency in cell population. In these cells, cell division may be inhibited by the ccd mechanism. The Ccd^+ Sop^- plasmids pXX318 and pXX344 are, therefore, more stably maintained than the Ccd^- Sop^- plasmid pXX327.

pXX325 and pXX326, which have the Sop^+ function but not the Ccd^+ function, are as stable as pSC138 (Fig. 12D). The effect of the ccd mechanism can not be clearly detected in Sop^+ mini-F plasmids as expected (3).

Incompatibilities of Mini-F

Mini-F plasmid pSC138 is known to be destabilized by the presence of another heterologous plasmid (for example, pBR322) carrying a segment of the mini-F plasmid (1). Incompatibilities of incB and incC, located in the essential region for replication of mini-F, are mediated by clusters of directly repeated nucleotide sequences of 19 or 22 bp (4,9,10) (see Fig. 1). As described above, the cis-acting sopC region shows an incompatibility. The incompatibility may correspond to the incD-mediated incompatibility in the IncFI group of plasmids (8) (Fig. 1 and 6). We have described another incompatibility located in the A2 segment. The functional region for the incompatibility, named incG, is located within the 47.3-48.7 kb segment (Fig. 1 and 6). The incG incompatibility was observed only under particular conditions as follows. When the A2 segment was cloned on pBR322, most of the plasmids carried the A2 segment with a deletion at the sopC region. When one of these pBR322 derivatives carrying the sopC-deleted A2 segment coexisted with pSC138, pSC138 was unstable and pSC138-derived plasmids with a deletion at sopC were selected during subsequent growth. The incG incompatibility may be caused by overproduction of SopB protein (Ref. 2; unpub. data).

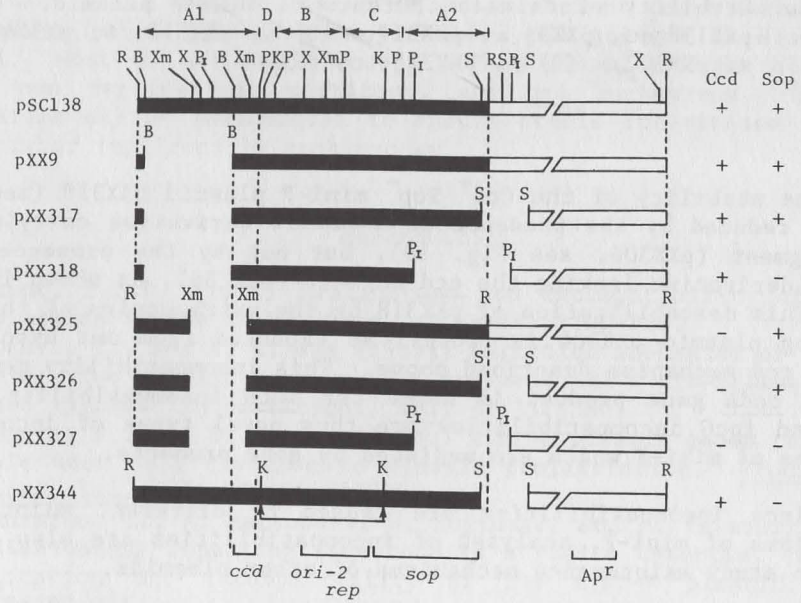


Fig. 11. Deletion mutants of mini-F plasmid pSC138. Restriction enzymes are shown in the legend of Fig. 3. B = BamHI, Xm = XmaI. In pXX344, the KpnI segment is inserted in the opposite direction.

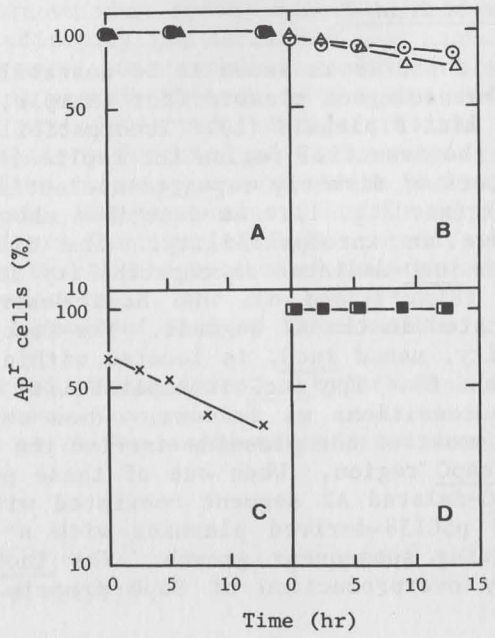


Fig. 12. Stability of deletion mutants of mini-F plasmid. (A) ●, pSC138; ○, pXX9; ▲, pXX317. (B) △, pXX318; ⊙, pX344. (C) ×, pXX327. (D) ■, pXX325; □, pXX326.

The stability of the Ccd$^+$ Sop$^-$ mini-F plasmid pXX318 (see Fig. 1) is reduced by the presence of a pBR322 derivative carrying the ccd segment (pXX306, see Fig. 10), but not by the presence of a pBR322 derivative lacking the ccd segment (pXX256), as shown in Fig. 13B. This destabilization of pXX318 by the extra copies of the ccd-carrying plasmid pXX306 is proper, as expected from our hypothesis on the ccd mechanism described above. This incompatibility mediated by the ccdA gene product is named the incH incompatibility. The incH and incG incompatibilities are thus novel types of incompatibilities of mini-F which are mediated by gene products.

Since incompatibilities are caused by different maintenance mechanisms of mini-F, analyses of incompatibilities are also important to study maintenance mechanisms of other plasmids.

SUMMARY

The F plasmid has its own partition mechanism and ccd mechanism (coupled cell division), besides its own replication mechanism, in order to be stably inherited into daughter cells through cell division. These 3 mechanisms are independent of one another. Therefore, when a DNA segment essential and sufficient for a mechanism is jointed to other heterologous plasmids, the segment is also functional. Most of natural low copy number plasmids might also have their own replication, partition, and ccd mechanisms. These mechanisms may be fundamental to ensure stable inheritance for low copy-number replicons in prokaryotes.

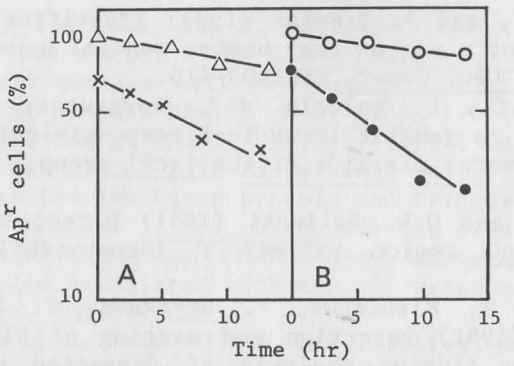


Fig. 13. Stability of mini-F plasmids. (A) Δ, mini-F pXX318, which carries both the ccdA and ccdB genes, but not the partition segment. x, mini-F pXX327, which carries neither the ccd genes nor the partition segment. (B) ●, stability of mini-F pXX318 in the presence of a pBR322 derivative carrying both the ccdA and ccdB genes (pXX306). ○, stability of mini-F pXX318 in the presence of a pBR322 derivative lacking the ccd genes (pXX256).

REFERENCES

1. Lane, H.E.D. (1981) Replication and incompatibility of F and plasmids in the incFI group. Plasmid 5:100-126.
2. Ogura, T., and S. Hiraga (1983a) Partition mechanism of F plasmid: Two plasmid gene-encoded products and a cis-acting region are involved in partition. Cell 32:351-360.
3. Ogura, T., and S. Hiraga (1983b) Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl Acad. Sci., USA 80:4784-4788.
4. Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami (1981) Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gen 15:257-271.
5. Watson, L.A., S.H. Phua, P.L. Bergquist, and H.E.D. Lane (1982) An Mr 29,000 protein is essential for mini-F maintenance in E coli. Gene 19:173-178.
6. Maki, S., M. Kuribayashi, T. Miki, and T. Horiuchi (1983) A amber replication mutant of F plasmid mapped in the minimal replication region. Mol. Gen. Genet. 191:231-237.

7. Kline, B.C., and J. Trawick (1983) Identification and characterization of a second copy number control gene in mini-F plasmids. Mol. Gen. Genet. 192:408-415.
8. Gardner, R.C., L. Malcolm, P.L. Bergquist, and H.E.D. Lane (1982) IncD, a genetic locus in F responsible for incompatibility with several plasmids of the incFI group. Mol. Gen. Genet. 188:345-352.
9. Tolun, A., and D.R. Helinski (1981) Direct repeats of the F plasmid incC region express F incompatibility. Cell 24: 687-694.
0. Komai, N., T. Nishizawa, Y. Hayakawa, T. Murotsu, and K. Matsubara (1982) Detection and mapping of six mini-F-encoded proteins by cloning analysis of dissected mini-F segments. Mol. Gen. Genet. 186:193-203.
1. Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsubara (1983) Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bact. 155:337-344.
2. Hiraga, S. (1976) Novel F prime factors able to replicate in Escherichia coli Hfr strains. Proc. Natl. Acad. Sci., USA 73:198-202.
3. Joh, K., and S. Hiraga (1979) Genetic mapping of the chromosomal replication origin of Salmonella typhimurium. J. Bact. 138:297-304.
4. Miki, T., S. Hiraga, T. Nagata, and T. Yura (1978) Bacteriophage λ carrying the Escherichia coli chromosomal region of the replication origin. Proc. Natl. Acad. Sci., USA 75:5099-5103.
5. Ogura, T., T. Miki, and S. Hiraga (1980) Copy-number mutants of the plasmid carrying the replication origin of the Escherichia coli chromosome: Evidence for a control region of replication. Proc. Natl. Acad. Sci., USA 77:3993-3997.
6. Austin, S., and A. Wierzbicki (1983) Two mini-F-encoded proteins are essential for equipartition. Plasmid 10:73-81.
7. Phua, S.H., P.L. Bergquist, and H.E.D. Lane (1982) Effects of Tn5 insertion in the incD region on mini-F maintenance and polypeptide synthesis. Mol. Gen. Genet. 188:353-355.
8. Nordström, K., S. Molin, and H. Aagaard-Hansen (1980) Partitioning of plasmid R1 in Escherichia coli. II. Incompatibility properties of the partitioning system. Plasmid 4:332-349.
9. Austin, S., and A. Abeles (1983) Partition of unit-copy miniplasmids to daughter cells. J. Mol. Biol. 169:373-387.
0. Mori, H., T. Ogura, and S. Hiraga (1984) Prophage induction caused by mini-F plasmid genes. Mol. Gen. Genet. (in press).
1. Bex, F., H. Karoui, L. Rokeach, P. Drèze, L. Garcia, and M. Couturier (1983) Mini-F encoded proteins: Identification of a new 10.5 kilodalton species. EMBO J. 2:1853-1861.
2. Karoui, H., F. Bex, P. Drèze, and M. Couturier (1983) Ham22, a mini-F mutation which is lethal to host cell and promotes recA-dependent induction of lambdoid prophage. EMBO J. 2:1863-1868.

23. Miki, T., K. Yoshioka, and T. Horiuchi (1984) Control of cel division by sex factor F in Escherichia coli. I. The 42.84 43.6 F segment couples cell division of the host bacteria wit replication of plasmid DNA. J. Mol. Biol. 174 (in press).
24. Miki, T., Z.T. Chang, and T. Horiuchi (1984) Control of cell division by sex factor F in Escherichia coli. II. Identification of genes for inhibitor protein and trigger protein on the 42.84-43.6 F segment J. Mol. Biol. 174 (in press).
25. D'Ari, R., and O. Huisman (1983) Novel mechanism of cell division inhibition associated with the SOS response in Escherichi coli. J. Bact. 156:243-250.
26. Borek, E., and A. Ryan (1960) The transfer of a biologicall active irradiation product from cell to cell. Biochim Biophys. Acta 41:67-73.

SEX PHEROMONES AND PLASMID TRANSFER IN *STREPTOCOCCUS FAECALIS*: A PHEROMONE, cAM373, WHICH IS ALSO EXCRETED BY *STAPHYLOCOCCUS AUREUS*

Don B. Clewell, Florence Y. An, Bryan A. White, and Cynthia Gawron-Burke

Departments of Oral Biology and Microbiology/Immunology
and The Dental Research Institute
Schools of Dentistry and Medicine
The University of Michigan
Ann Arbor, Michigan 48109

INTRODUCTION

Recipient strains of *Streptococcus faecalis* excrete small, peptide sex pheromones which induce donors harboring certain conjugative plasmids to synthesize a proteinaceous adhesin that facilitates the formation of mating aggregates. (For a recent review of this phenomenon, see Ref. 2.) Referred to as "aggregation substance," the adhesin has been revealed as a unique antigen that uniformly coats the donor cell surface (14,25). Self aggregation of donors also occurs; for this reason the pheromones are also referred to as clumping inducing agents (CIAs). It has been proposed that "aggregation substance" binds specifically to a "binding substance" present on the surface of both donor and recipient cells (6,7).

Upon acquiring a copy of the plasmid, the recipient ceases to excrete the related pheromone, but pheromones specific for donors harboring unrelated classes of conjugative plasmids continue to be excreted (5,7). A specific sex pheromone, therefore, is defined by the plasmid system being used to resolve it. The activity designated cAD1 represents the pheromone to which strains harboring pAD1 respond; similarly, donors harboring pPD1, pAMγ2, etc., respond, respectively, to cPD1, cAMγ2, etc.

The "shut-off" of pheromone synthesis in donor cells is accomplished by a plasmid-encoded inactivation of the peptide via a chemical addition, the nature of which is unknown (12). A phosphodiester bond is generated in the process, and the modified pheromone

is capable of inhibiting exogenous pheromone activity--probably by competition for a pheromone receptor site. On the basis of gel filtration chromatography, the pheromones cAD1 and cPD1 were roughly sized at 1,050 and 800 daltons, respectively, and the corresponding modified forms (designated mcAD1 and mcPD1, respectively) appear to be 300-400 d larger (12). Purification of the peptides has been difficult because of the extremely low concentrations in filtrates. However, Suzuki and coworkers have recently been successful in the purification of cPD1; the peptide was found to consist of 8 amino acids and is very hydrophobic (A. Suzuki, pers. comm.). The molecular weight is 912.

Mutants significantly reduced in the production of cPD1 (i.e., to 3-4% that of the wild-type) have been derived and found to also be reduced in cAD1 production and probably additional pheromones (2,12). These variants were poor recipients; however, those which acquired plasmid DNA (e.g., pAM714, a derivative of pAD1) were good donors. Indeed, since the latter also failed to excrete the modified pheromone (i.e., mcAD1), with its attendant inhibitory effects, donor sensitivity to exogenous pheromone was greater than normal (2). The reduction of more than one pheromone activity in a single mutation raises the possibility that synthesis or subsequent processing or transport of these substances is linked.

The genetics of the pAD1 pheromone response have been partially analyzed using the erythromycin (Em)-resistance transposon Tn*917* (11). Two regions on the plasmid, designated *traA* (1.5 kb) and *traB* (1.3 kb) and separated by 1.7 kb, appear to be involved in a negative regulation of the synthesis of "aggregation substance"; insertions into these regions give rise to constitutive aggregation and the ability to donate at high frequency in short (10 min) matings. Other mutants, exhibiting a significantly reduced mating potential, were also derived (11).

CIA ACTIVITIES PRODUCED BY SPECIES OTHER THAN *STREPTOCOCCUS FAECALIS*

Although extracellular compounds that affect competence for transformation are well known in *Streptococcus pneumoniae* and *Streptococcus sanguis* (15), substantive evidence for bacterial sex pheromones which promote conjugative DNA transfer has been reported only in *Streptococcus faecalis*. To determine if different species of bacteria might excrete substances similar to the *S. faecalis* sex pheromones, 10 *S. faecalis* strains exhibiting high sensitivity to induced clumping by plasmid-free *S. faecalis* filtrates were used to screen filtrates from a variety of other bacterial sources. (Relevant strains and plasmids used in this study are listed in Tab. 1 and 2.) Table 3A shows results for 4 of these responder strains, 3 of which (39-5, DS16, and DS5) have been previously characterized

Tab. 1. Bacterial strains used in this study.

Strain	Relevant Chromosome markers	Plasmid Content	Source
Strep. faecalis			
JH2-2	*rif*, *fus*	none	(13)
FA2-2	*rif*, *fus*	none	(3)
JH2SS	*str*, *spc*	none	(22)
FA1001	*rif*, *fus*	none	This study; (derivative of JH2-2)
Y11	*str*, *spc*, *tet*(Tn*916*)	none	(12)
39-5		pPD1, pPD2, pPD3, pPD4, pPD5, pPD6	(25)
DS16	*tet*(Tn*916*)	pAD1, pAD2	(3, 23)
DS5	*tet*	pAMγ1, pAMγ2, pAMγ3 pAMβ1, pAMα1	(4, 5)
RC73	*tet*(Tn*918*) (probable chromosomal location)	pAM373, at least four additional plasmids	This study; University Hospital, Ann Arbor
FA373	*rif*, *fus*, *tet*(Tn*918*)	pAM373	This study
FA381	*str*, *spc*, *tet*(Tn*918*)	none	This study
CG110	*rif*, *fus*, *tet*(Tn*916*)	none	(9)
Strep. faecium			
9790(ATCC)		cryptic plasmids present	G. Shockman
Strep. sanguis			
Challis (NCTC 7868)(V288)		none	F. Macrina
Challis RF	*rif*, *fus*	none	This study
G9B		none	(18), Garth Jones
Staph. aureus			
879R4	(restriction negative)	12.5 kb cryptic plasmid	(20) P.Pattee
879R4S	*str* (derivative of 879R4)	"	This study
879R4RF	*rif*, *fus* (derivative of 879R4)	"	This study
RN450		none	(17), R. Novick
Escherichia coli			
CG170	DH1 background	pAM170	(10)
FA390	DH1 background	pAM390	This study

Tab. 2. Relevant plasmids used in this study.

Plasmid	Marker	Comment
pAD1	Hem-Bac	57 kb, conjugative plasmid from S. faecalis DS16
pAM373	none	36 kb, conjugative plasmid from S. faecalis RC73
pAM377	Em^r(Tn917)	pAM373 with Tn917 insertion
pAM378	Tc^r(Tn918)	pAM373 with Tn918 insertion
pAM380	Em^r(Tn917); Tc^r(Tn916)	pAM377 with Tn916 insertion
pAM81::Tn917	Tc^r, Em^r(Tn917)	non-conjugative; mobilized easily; see reference 1
pAM180	Em^r, Tc^r(Tn916)	a derivative of the 16 kb, conjugative, Em^r plasmid pAM81 with Tn916 insertion; see reference 9
pAM170	Ap^r, Tc^r(Tn916)	E. coli plasmid vector pGL101 with a cloned fragment carrying Tn916; see reference 10
pAM390	Ap^r, Tc^r(Tn918)	E. coli plasmid vector pGL101 with a cloned fragment carrying Tn918

with respect to their plasmid content (3,4,5,23,25). The 3 latter strains, as well as the 6 S. faecalis strains not shown (DU9, RC11, RC26, RC48, RC56, and RC87), all failed to respond to any of the filtrates. Interestingly, strain RC73 responded to filtrates from Strep. faecium (strain 9790), Strep. sanguis (strains Challis and G9B), and Staphylococcus aureus (strains 879R4S and RN450). Filtrates from the following bacteria (not shown in Tab. 3a) exhibited no CIA activity for any of the 10 responders: Strep. sanguis A1, Strep. pyogenes AC1, Strep. agalactiae T-2, Strep. lactis 4027, Strep. cremoris 4158, Strep. milleri CRS8507, Staph. epidermidis JM899, Bacillus subtilis BR151, and Escherichia coli DH1. It was also of interest that 2 mutants of S. faecalis (FA1001 and Y11) that were defective in cPD1 and cAD1 activity, and whose filtrates generated little or no response by 39-5, DS16, or DS5, produced essentially unaltered levels of CIA activity to which RC73 responded (Tab. 3B).

The response of RC73 to the 2 Staph. aureus filtrates prompted an examination of additional clinical isolates of Staph. aureus as

Tab. 3. CIA titers of culture filtrates from different species.

			S. faecalis responders			
			39-5	DS16	DS5	RC73
A.						
	Strep. faecalis	JH2-2	64	32	64	128
	Strep. faecium	9790	<2	<2	<2	8
	Strep. sanguis	Challis	<2	<2	<2	16
		G9B	<2	<2	<2	8
	Staph. aureus	879R4S	<2	<2	<2	32
		RN450	<2	<2	<2	32
B.						
	Strep. faecalis	JH2-2	64	32	64	128
		FA1001	2	<2	<2	64
		Y11	2	<2	2	128

a Titers were measured using a microtiter dilution assay as described in reference 7. The values represent the reciprocal of the highest serial (two fold) dilution which still exhibited a detectable clumping response using the indicated responder strain.

A. Filtrates of selected strains of different species containing CIA activity detected by at least one S. faecalis responder strain.

B. Comparisons of filtrates from S. faecalis variants reduced in production of cAD1 and cPD1. FA1001 was generated by mutagenesis of JH2-2 with "nitrosoguanidine" using the same screening method used to obtain Y11 (see reference 12).

well as its less pathogenic, coagulase negative counterparts (e.g., Staph. epidermidis). All of 21 additional strains of Staph. aureus examined excreted activities (titers of 16-64) to which RC73 responded (data not shown). This was in contrast to 22 isolates of coagulase negative staphylococci among which only 2 excreted such an activity. (In the 2 positive cases, the titers were only 2.)

The production of CIA activity by Strep. sanguis is not as prevalent as was found for Staph. aureus. Whereas strains Challis and G9B excreted significant activity, several other Strep. sanguis strains tested did not.

IDENTIFICATION OF THE PLASMID IN S. FAECALIS RC73 ENCODING THE RELATED CIA RESPONSE

The apparent correlation of a CIA activity with staphylococcal pathogenicity stimulated further analyses of the responding system. S. faecalis RC73 is hemolytic, bacteriocinogenic, and resistant to tetracycline; sedimentation and electrophoretic analyses revealed at least 5 plasmids -- the largest being about 57 kb. In order to derive strains harboring only one or a few of these plasmids, matings were performed between RC73 and the plasmid-free S. faecalis strain FA2-2. Selection for Tc-resistant transconjugants from overnight filter matings (8) yielded transconjugants at a frequency of about 10^{-6} per donor (about 10% were hemolytic), and these contained a variety of plasmid combinations. It became evident that only strains harboring a 36-kb plasmid to be designated pAM373 exhibited a response to filtrates of Staph. aureus and Strep. sanguis; indeed, one transconjugant (FA373) harbored only this plasmid. A restriction map of pAM373 is shown in Fig. 1. The plasmid is maintained at about 4 copies per chromosome and does not bear the RC73 determinants for hemolysin, bacteriocin, or Tc-resistance. (The nature of the Tc-resistance determinant selected for in the mating experiments is discussed below.)

To serve as a useful marker, the 5 kb Em-resistance transposon Tn917 (3,21,22) was introduced onto pAM373 using the following approach. Strain FA373, which harbors pAM373 as its only plasmid, was mated (overnight on filters at a donor to recipient ratio of 1:1) with S. faecalis JH2SS (pAMα1::Tn917). [pAMα1 is a 9-kb, nonconjugative Tc-resistance plasmid (1), known to be readily mobilizable by several different conjugative plasmids.] The mating mixture was plated on media containing rifampin, fusidic acid (for donor selection) and Em in order to select for transconjugants in which pAM373 transferred to the recipient and mobilized back the pAMα1::Tn917 plasmid to donors. Transconjugants arose at approximately 10^{-8} per donor; of these 1 out of 3 tested transferred Em-resistance at high frequency without cotransfer of Tc-resistance. Subsequent analyses showed that Tn917 had inserted into pAM373, probably during the initial mobilization event. The resulting plasmid was designated pAM377, and it transferred at high frequency (10^{-3}) in 4-hr broth matings. As shown in Fig. 2A (lane 2), an insertion (5 kb) into the EcoR1 A fragment had occurred.

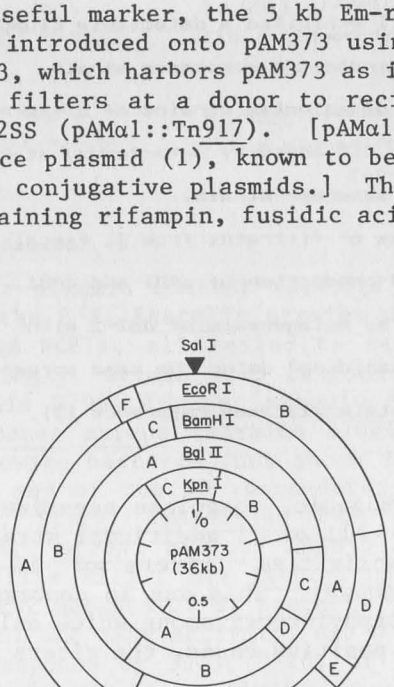


Fig. 1. Restriction map of pAM373. Mapping was based on analyses of double and partial digestions with appropriate enzymes.

An additional derivative of pAM373, having both Em- and Tc-resistance determinants, was generated by inserting the conjugative transposon Tn916 (Tc) onto pAM377 (Em) using an approach previously described (8,9). Briefly, pAM377 was introduced into S. faecalis CG110 (harbors Tn916 on the chromosome); use of the resulting derivative as a donor in subsequent matings that selected for transfer of Tc-resistance gave rise to a pAM377 derivative carrying Tn916. Designated pAM380, the plasmid acquired the insertion in its EcoR1 A fragment, which already contained Tn917 (Fig. 2A, lane 4). Further analyses (data not shown) have shown that Tn916 is actually inserted into a Bgl II fragment within Tn917.

Interestingly, the Tc-resistance determinant selected for in the initial RC73 x FA2-2 matings proved to be chromosome-borne; indeed, subsequent matings yielded a few Tc-resistant transconjugants found to be plasmid-free. Matings using strain FA373 (Tc-resistant, yet harboring only pAM373) as a donor and JH2SS as a recipient yielded Tc-resistant transconjugants at a frequency of 10^{-5}; and of 14 isolates tested for donor potential in cross-streak matings (3), 8 were found to transfer Tc-resistance at high frequency. Plasmid DNA from 3 of these strains was examined and found to contain a segment of DNA inserted into the EcoR1 fragment C of pAM373. A restriction digest of one of these, designated pAM378, is shown in Fig. 2A (lane 3). (The inserted DNA did not contain an EcoR1 site.)

The Tc-resistance determinant was also capable of inserting into other plasmids. When the hemolysin plasmid pAD1 was introduced into a plasmid-free Tc-resistant derivative (FA381; a derivative of JH2SS) and subsequent matings were performed using FA2-2 as a recipient, Tc-resistant transconjugants (of which 60% were hemolytic) with the resistance marker linked to pAD1 could be generated at a frequency of about 10^{-8} (about 10-20% of the hemolytic transconjugants). Figure 2B (lanes 2 and 3) shows examples of insertions into 2 different EcoR1 restriction fragments (B and D), one of which (D) gave rise to a hyperhemolytic phenotype. The enhanced hemolysin expression resembled a phenomenon known to occur in the case of some Tn916 insertions into pAD1 (8).

Finally, it is noted that among the transconjugants of the original mating between RC73 and FA2-2, a hemolytic derivative subsequently used as a donor in matings with JH2SS gave rise to Tc-

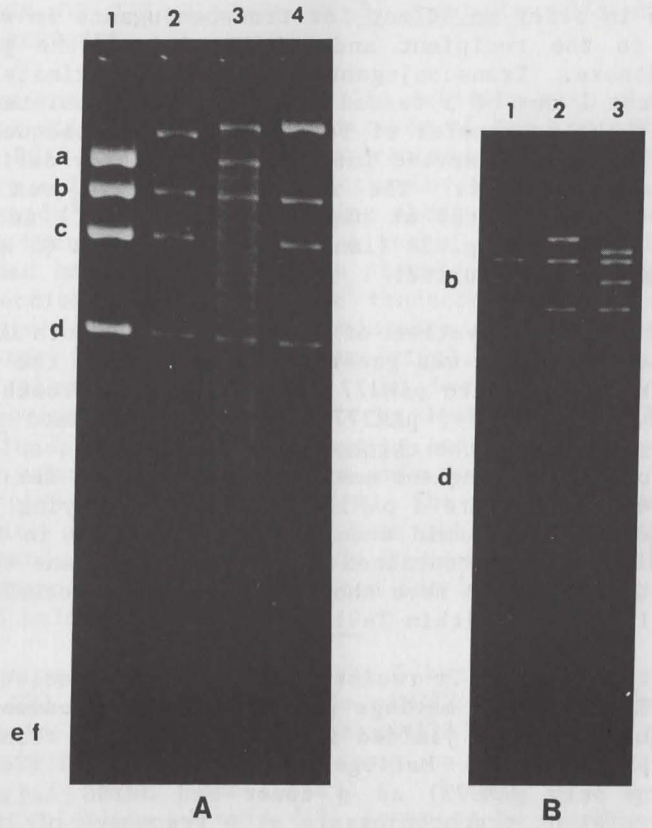


Fig. 2. Agarose gel electrophoresis of plasmid DNA digested with EcoR1. The procedure was as described elsewhere (8). A. pAM373 (lane 1); pAM377 (lane 2); pAM378 (lane 3); pAM380 (lane 4). The lower case letter on the left corresponds to the EcoR1 fragment of pAM373 (fragments e and f migrate together). B. pAD1 (lane 1); pAD1::Tn918 (lane 2) (insertion in b fragment); pAD1::Tn918 (lane 3) (insertion in d fragment). The b and d fragments of pAD1 are indicated on the left.

resistant, hemolytic strains of which about 10% were hyperhemolytic. In these cases the Tc-resistance determinant was linked to a 57-kb hemolysin plasmid originally present in RC73.

In all of the cases where it was measured (e.g., those shown in Fig. 2), the size of the inserted DNA associated with Tc-resistance was estimated at ∿15kb (similar to that of Tn916). The element will henceforth be designated Tn918.

ON THE TRANSFERABILITY OF pAM373

As shown in Tab. 4, the pAM373 derivatives pAM377(Em) and pAM378(Tc) transferred efficiently in 4-hr broth matings between strains of S. faecalis; under the same conditions, however, no transfer could be observed from S. faecalis to either Strep. sanguis Challis or the restriction negative (20) Staph. aureus 879R4RF. In further efforts to transfer plasmid DNA to these species, overnight filter matings were performed. In the case of pAM377 (Em), transfer of Em-resistance to Staph. aureus or Strep. sanguis could not be detected; however, when pAM378(Tc) was used, Tc-resistant transconjugants appeared at 3×10^{-5} among Staph. aureus and at a very low frequency (4×10^{-9}) among Strep. sanguis. When several of these transconjugants were examined for plasmid content, pAM378 could not

Tab. 4. Mating experiments α.

Donor	Recipient	Selection	Frequency. Transconjugants per donor
A. Broth matings (4 hrs)			
JH2SS(pAM377)	FA2-2	Rif,Fa,Em	5.9×10^{-3}
"	879R4RF	Rif,Fa,Em	$<3 \times 10^{-9}$
"	Challis RF	Rif,Fa,Em	$<5 \times 10^{-9}$
JH2SS(pAM378)	FA2-2	Rif,Fa,Tc	3.0×10^{-2}
"	879R4RF	Rif,Fa,Tc	$<1 \times 10^{-9}$
"	Challis RF	Rif,Fa,Tc	$<1 \times 10^{-9}$
B. Filter matings (overnight)			
JH2SS(pAM377)	FA2-2	Rif,Fa,Em	4.6×10^{-2}
"	879R4RF	Rif,Fa,Em	$<2 \times 10^{-9}$
"	Challis RF	Rif,Fa,Em	$<2 \times 10^{-9}$
JH2SS(pAM378)	FA2-2	Rif,Fa,Tc	3.3×10^{-2}
"	879R4RF	Rif,Fa,Tc	3.0×10^{-5}
"	Challis RF	Rif,Fa,Tc	4×10^{-9}
FA2-2(pAM380)	879R4S	Em,Sm	$<1 \times 10^{-9}$
"	"	Tc,Sm	1×10^{-7}
JH2-2(pAM180)	879R4S	Em,Sm,	1×10^{-7} (6% Tc^r)
"	"	Tc,Sm	1×10^{-7} (83% Em^r)

a Mating and plating procedures were as described elsewhere (7, 8). Rif, rifampin; Fa, fusidic acid.

be found. (Only a 13.5-kb cryptic plasmid already present in the staphylococcal recipient was evident.) In the case of pAM380 (pAM373::Tn917::Tn916), Tc-resistant transconjugants arose at 10^{-8}; whereas Em-resistant transconjugants could not be obtained. Those Tc-resistant transconjugants that were obtained were sensitive to Em, and no new plasmid could be detected.

The establishment of the Tn918 and Tn916 markers in Staph. aureus is consistent with a behavior previously observed for Tn916 in S. faecalis (9). When located on a conjugative plasmid such as pAM81 (24 kb, Em-resistance), Tn916 appears to undergo a "zygotic induction" when introduced into a new strain. That is, following transfer of pAM180 (pAM81::Tn916), Tn916 excises from the plasmid and is either lost or inserts into the chromosome. When pAM180 was used in matings with Staph. aureus (Tab. 4), Tc-resistant transconjugants as well as Em-resistant transconjugants could be obtained; however, not all Em-resistant transconjugants were Tc-resistant and vice versa. This is similar to the behavior previously observed (9) in matings between S. faecalis strains. In view of the multiple similarities between Tn918 and Tn916, it seemed likely that transfer of Tc-resistance from pAM378 donors resulted in insertions of Tn918 into the recipient chromosome. Since the frequency of Tc-resistant transconjugants was relatively high ($\sim 10^{-5}$), it is conceivable that the plasmid actually entered the staphylococcal recipient but could not be maintained and was subsequently segregated.

FURTHER ANALYSES OF TN918 AND COMPARISONS TO TN916

It has been possible to clone Tn918 in Escherichia coli DH1 by ligating a pAD1 EcoR1 D::Tn918 fragment into the single EcoR1 site of pGL101 [ampicillin resistance (Ap), 2.4 kb] (16). E. coli transformants resistant to both Ap and Tc could be obtained; however, Tc-resistance was lost at high frequency (e.g., over 90% of the cells became sensitive to Tc during unselected overnight growth) without loss of Ap-resistance. Analyses of the sensitive derivatives have shown that Tn918 was lost and that there was a regeneration of the EcoR1 D fragment (i.e., the pAD1 fragment into which Tn918 had been inserted). Exactly the same phenomenon had been previously observed for Tn916 (10). When a Tn918 clone designated pAM390 was compared with a similar Tn916 clone (pAM170), restriction digests revealed 5 similar HincII fragments (within the transposons) and single recognition sites for HindIII and KpnI (data not shown). As expected, strong homology was evident between Tn916 and Tn918 in Southern blot hybridizations (19) using Tn916 as a probe.

ANALYSIS OF THE TN918 AND TN916 INSERTS IN STAPH. AUREUS

Tn916 has been previously shown to insert into a number of different sites in the S. faecalis chromosome; this could be demonstrated by hybridizing a ^{32}P-labeled restriction fragment containing Tn916 to chromosomal DNA cleaved with HindIII (9). In the case of each insert, 2 fragments hybridized; these represented the 2 transposon-host DNA junction fragments extending from the single HindIII site within Tn916. Because the size of these fragments differed in the various isolates, insertions into different sites must have occurred. Figure 3 shows the results of similar experiments in which ^{32}P-labeled pAM170 (a pGL101 chimera containing Tn916) was hybridized to HindIII digested chromosomal DNA isolated from several staphylococcal strains containing Tn918 or Tn916 inserts. The transposons had been introduced from their positions on plasmid DNA (i.e., from pAM378 or pAM380; see above). It is readily apparent that insertions of both Tn918 and Tn916 occurred into different sites on the recipient chromosome; and in a few cases more than one transposon insertion was evident.

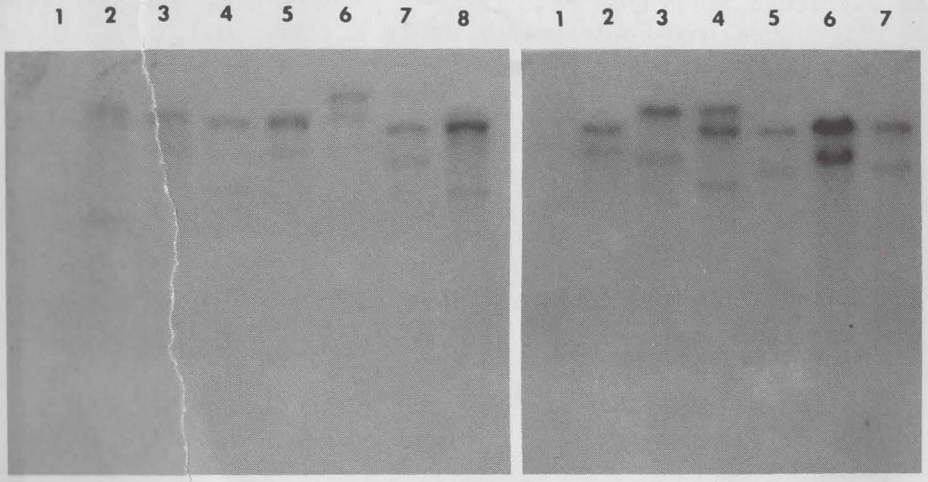


Fig. 3. Autoradiograms obtained from filter blot of HindIII-digested chromosomal DNA of Tc-resistant transconjugants of Staph. aureus and hybridization to ^{32}P-labeled pAM170 (contains Tn916). The method was that of Southern (19) as modified by Wahl et al. (24). A. Lanes 2 through 8 represent 7 different transconjugants from matings between S. faecalis JH2SS(pAM378) and Staph. aureus 879R4RF. Lane 1 represents a Tc-sensitive recipient (879R4RF). B. Lanes 2 through 7 represent transconjugants acquiring Tn916 as a result of matings between S. faecalis FA2-2 (pAM380) and Staph. aureus 879R4S. Lane 1 is a Tc-sensitive recipient (879R4S).

strated by hybridizing a ^{32}P-labeled restriction fragment containing Tn916 to chromosomal DNA cleaved with HindIII (9). In the case of each insert, 2 fragments hybridized; these represented the 2 transposon-host DNA junction fragments extending from the single HindIII site within Tn916. Because the size of these fragments differed in the various isolates, insertions into different sites must have occurred. Figure 3 shows the results of similar experiments in which ^{32}P-labeled pAM170 (a pGL101 chimera containing Tn916) was hybridized to HindIII digested chromosomal DNA isolated from several staphylococcal strains containing Tn918 or Tn916 inserts. The transposons had been introduced from their positions on plasmid DNA (i.e., from pAM378 or pAM380; see above). It is readily apparent that insertions of both Tn918 and Tn916 occurred into different sites on the recipient chromosome; and in a few cases more than one transposon insertion was evident.

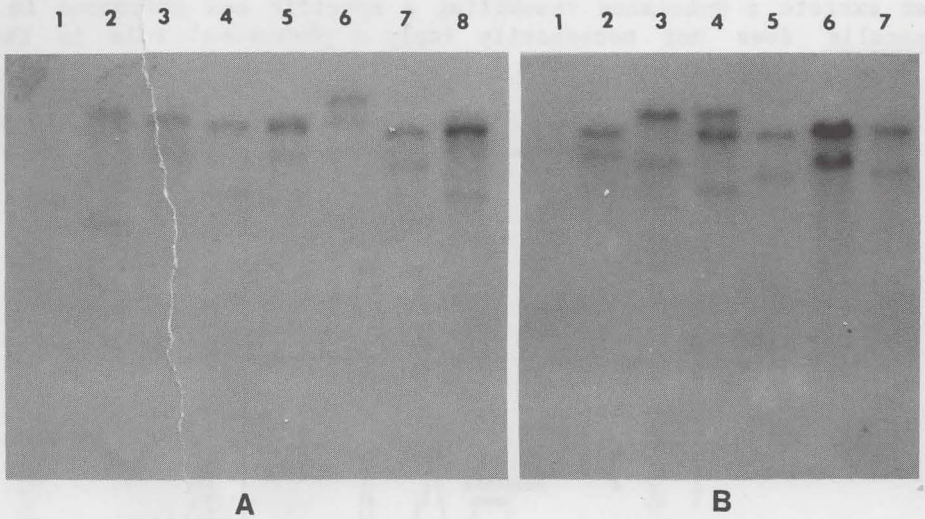


Fig. 3. Autoradiograms obtained from filter blot of HindIII-digested chromosomal DNA of Tc-resistant transconjugants of Staph. aureus and hybridization to ^{32}P-labeled pAM170 (contains Tn916). The method was that of Southern (19) as modified by Wahl et al. (24). A. Lanes 2 through 8 represent 7 different transconjugants from matings between S. faecalis JH2SS(pAM378) and Staph. aureus 879R4RF. Lane 1 represents a Tc-sensitive recipient (879R4RF). B. Lanes 2 through 7 represent transconjugants acquiring Tn916 as a result of matings between S. faecalis FA2-2 (pAM380) and Staph. aureus 879R4S. Lane 1 is a Tc-sensitive recipient (879R4S).

PROPERTIES OF THE PHEROMONE cAM373

Similar to the cases of cAD1 and cPD1, the pheromone that induces pAM373 transfer, now referred to as cAM373, was heat stable, resistant to trypsin, and sensitive to chymotrypsin. The results of gel filtration chromatography are shown in Fig. 4A, where cAM373 is observed to elute between cAD1 and cPD1. On the basis of the previously estimated sizes of cAD1 and cPD1 (12), cAM373 was approximated at 850 d.

It was also found that strains harboring pAM373 or related derivatives excreted low levels of an inhibitor activity presumed to represent a modified form of the pheromone. Designated mcAM373, this activity was observed to have a mass about 300-400 d larger than cAM373 (Fig. 4B).

CONCLUDING REMARKS

The observation that members of a few other bacterial species can excrete a substance resembling a specific sex pheromone in S. faecalis does not necessarily imply a pheromonal role in those

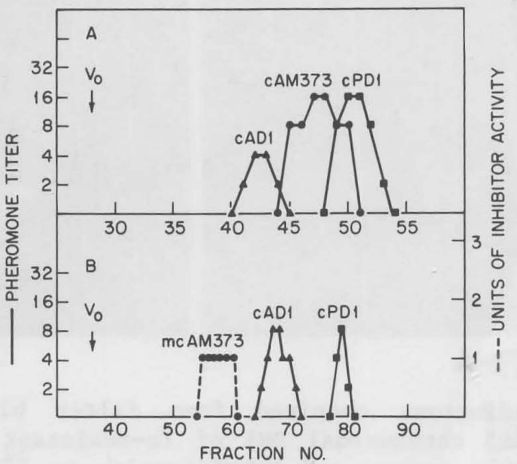


Fig. 4. Gel filtration chromatography of culture filtrates of S. faecalis strains. Fractionation on a Bio-Rad P2 polyacrylamide column (200-400 mesh; 1.6 x 100 cm) and the corresponding assays were as described elsewhere (12). Vo refers to the void volume. Strain FA373 was used as a responder to measure cAM373 activity. (A) Filtrate of JH2-2. (B) Filtrate of FA2-2(pAM378). (Note: a unit of inhibitor is defined as the amount capable of giving rise to a 50% reduction of pheromone activity.

species. Indeed, the fact that pAM373 derivatives could not be established in Staph. aureus or Strep. sanguis suggests the possible absence of a direct relationship between cAM373 and mating in these organisms. As previously noted (1), it is conceivable that bacterial production of the substances which donor strains recognize as sex pheromones could have preceded the evolution of the related conjugative systems, with the latter simply taking advantage of these available, extracellular compounds as mating signals. If this is true, the question arises as to the nature of the original, perhaps continuing, function of these peptides. The production of cAM373 activity by all Staph. aureus strains tested, and a general absence of the activity in most coagulase-negative staphylococci, raises the possibility of a role in virulence. The derivation of mutants defective in cAM373 production and their examination in virulence assays could shed light on this interesting question.

Although pAM373 derivatives failed to establish in Staph. aureus, the fact that they could facilitate insertions of Tn918 and Tn916 at multiple sites in the staphylococcal chromosome suggests their potential as delivery vehicles for these transposons in this species. After targeting a particular gene by mutagenic insertion, restriction fragments containing either of these transposons could be cloned in E. coli (10) where excision then gives rise to a regenerated segment of DNA containing the specific staphylococcal locus of interest.

ACKNOWLEDGMENTS

We wish to thank M. Smith, Y. Yagi, E. Ehrenfeld, G. Fitzgerald, Y. Ike, R. Wirth, J. Shaw, and R. Kessler for helpful discussions. We also thank M. O'Connor-Scarlet and our many colleagues for their generous donations of bacterial strains. This work was supported by National Institutes of Health grants DE02731 and AI10318. C.G.-b is the recipient of a Junior Faculty Research Award from the American Cancer Society, and B.W. is supported by a National Institute of Health Postdoctoral Fellowship.

REFERENCES

1. Clewell, D. (1981) Plasmids, drug resistance, and gene transfer in the genus Streptococcus. Microbiol. Rev. 45:409-436.
2. Clewell, D.B., B.A. White, Y. Ike, and F. An (1984) Sex pheromones and plasmid transfer in Streptococcus faecalis. In Microbial Development, R. Losick and L. Shapiro, eds. Cold Spring Harbor Press, Cold Spring Harbor, New York, pp. 122-149.
3. Clewell, D., P. Tomich, C. Gawron-Burke, A. Franke, Y. Yagi, and F. An (1982) Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. J. Bact. 152:1220-1230.

4. Clewell, D., Y. Yagi, G. Dunny, and S. Schultz (1974) Characterization of three plasmid DNA molecules in a strain of Streptococcus faecalis. Identification of a plasmid determining erythromycin resistance. J. Bact. 117:283-289.
5. Clewell, D., Y. Yagi, Y. Ike, R. Craig, B. Brown, and F. An (1982) Sex pheromones in Streptococcus faecalis: Multiple pheromone systems in strain DS5, similarities of pAD1 and pAMγ1, and mutants of pAD1 altered in conjugative properties. In Microbiology-1982, D. Schlessinger, ed. American Soc. Microbiol., Washington, D.C. pp. 97-100.
6. Dunny, G., B. Brown, and D. Clewell (1978) Induced cell aggregation and mating in Streptococcus faecalis. Evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci., USA 75:3479-3483.
7. Dunny, G., R. Craig, R. Carron, and D. Clewell (1979) Plasmid transfer in Streptococcus faecalis. Production of multiple sex pheromones by recipients. Plasmid 2:454-465.
8. Franke, A., and D. Clewell (1981) Evidence for a chromosome-borne resistance transposon in Streptococcus faecalis capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bact. 145:494-502.
9. Gawron-Burke, C., and D. Clewell (1982) A transposon in Streptococcus faecalis with fertility properties. Nature 300:281-284.
10. Gawron-Burke, C., and D. Clewell (1984) Regeneration of insertionally inactivated streptococcal DNA fragments following excision of Tn916 in Escherichia coli: Strategy for targeting and cloning of genes from gram-positive bacteria. J. Bact. 159:214-221.
11. Ike, Y., and D. Clewell (1984) Genetic analysis of the pAD1 pheromone response in Streptococcus faecalis using Tn917 as an insertional mutagen. J. Bact. 158:777-783.
12. Ike, Y., R. Craig, B. White, Y. Yagi, and D. Clewell (1983) Modification of Streptococcus faecalis sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci., USA 80:5369-5373.
13. Jacob, A., and S. Hobbs (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var zymogenes. J. Bact. 117:360-372.
14. Kessler, R., and Y. Yagi (1983) Identification and partial characterization of a pheromone-induced adhesive surface antigen of Streptococcus faecalis. J. Bact. 155:714-721.
15. Lacks, S. (1977) Binding and entry of DNA in bacterial transformation. In Microbial Interactions, Series B, Receptors and Recognition, Vol. 3. J.L. Reissig, ed. Chapman and Hall, London, pp. 177-232.
16. Lauer, G., R. Pastrana, J. Sherley, and M. Ptashne (1981) Construction of overproducers of the bacteriophage 434 repressor and cro proteins. J. Molec. and Appl. Genet. 1:139-147.

17. Novick, R., and R. Brodsky (1972) Studies on plasmid replication. 1. Plasmid incompatibility and establishment in Staphylococcus aureus. J. Molec. Biol. 68:285-302.
18. Rosan, B. (1976), Relationship of the cell wall composition of group H streptococci and Streptococcus sanguis to their serological properties. Infect. and Immun. 13:1144-1153.
19. Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol. 98:503-517.
20. Stobberingh, E., and K. Winkler (1977) Restriction-deficient mutants of Staphylococcus aureus. J. Gen. Microbiol. 99:359-367.
21. Tomich, P., F. An, and D. Clewell (1978) A transposon (Tn917) in Streptococcus faecalis which exhibits enhanced transposition during induction of drug resistance. Cold Spring Harbor Symp. Quant. Biol. 43:1217-1221.
22. Tomich, P., F. An, and D. Clewell (1980) Properties of erythromycin-inducible transposon Tn917 in Streptococcus faecalis. J. Bact. 141:1366-1374.
23. Tomich, P., F. An, S. Damle, and D. Clewell (1979) Plasmid related transmissibility and multiple drug resistance in Streptococcus faecalis subsp. zymogenes strain DS16. Antimicrob. Ag. Chemother. 15:828-830.
24. Wahl, G., M. Stern, and G. Stark (1979) Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci., USA 76:3683-3687.
25. Yagi, Y., R. Kessler, J. Shaw, D. Lopatin, F. An, and D. Clewell (1983) Plasmid content of Streptococcus faecalis strain 39-5 and identification of pheromone (cPD1)-induced surface antigen. J. Gen. Microbiol. 129:1207-1215.

GENERAL GENETIC RECOMBINATION OF BACTERIAL PLASMIDS

Amikam Cohen, Zipora Silberstein,
Shimshon Broido, and Avraham Laban

Department of Molecular Genetics
Hadassah Medical School
The Hebrew University
P.O. Box 1172
Jerusalem 91-101, Israel

PLASMID RECOMBINATION

Analysis of genetic recombination between 2 chromosomes, which differ from each other by at least 2 genetic markers, is based mainly on the production of recombinants which are phenotypically distinguishable from their progenitors. The use of small DNA molecules such as bacterial plasmids as recombination substrates provides an additional handle for the investigation of recombination. It also allows the description of recombination events in molecular terms. Recombination of 2 plasmids, genetically distinguishable from each other, can be followed as recombination reactions in other systems by monitoring changes in the phenotypic expression of the plasmid genes (1,2,3). But in addition, plasmidic recombination may be investigated by analyzing changes of their molecular structures. Interplasmidic recombination leads to the formation of plasmid oligomers and intraplasmidic recombination between repeated sequences can lead to deletion of the region between the crossing-over sites, and, when the recombination substrate is an oligomer, to monomerization. When mutations affect restriction endonuclease sites, recombination products may be compared to recombination substrates by restriction endonculease analysis (2,4,5). The small size of bacterial plasmids also allows the isolation and characterization of molecular intermediates of the recombination pathway (6,7).

These features of the plasmid recombination system allow determination of the dependency of plasmid recombination on host gene functions (1,2,3,6,7,8), isolation of recombinogenic elements that increase frequency of recombination (9), determination of the length of homology which is involved in recombination (10,11), and investigation of the molecular nature of the recombination pathway (4,5,6).

While plasmids are preferred probes for the investigation of bacterial recombination, their susceptibility to the bacterial system is of major concern to those who are interested in molecular cloning of DNA. Recombination reactions which affect plasmid molecular structure may also interfere with the integrity of cloned DNA fragments, especially when these fragments contain repetitive sequences. Loss of DNA sequences by intraplasmidic recombination has affected several cloned DNA fragments, including highly repeated satellite Drosophila DNA (12), yeast ribosomal DNA (13,14) adenovirus DNA (15), balbini ring DNA (16), retrovirus-like repeated mouse gene family (17), and Moloney sarcoma virus DNA (18). Attempts have been made to overcome the problem of the instability of cloned DNA by using recA mutants as recipient bacteria. However, since plasmid recombination reactions are at least partially recA-independent (2,3,4,11,14,15) this approach has limited success.

The length of the repeated DNA sequence and the degree of homology determine recombination frequency. Frequencies of 10^{-3} to 10^{-4} recombinants per cell have been observed when recombination occurred between homologous sequences of several hundred nucleotides in rec^+ hosts (2,3). When very short direct repeats are involved (5 to 7 nucleotides), frequencies of 10^{-7} to 10^{-8} deletions per cell have been determined (10,11). It has not yet been established whether the same mechanism is involved in the 2 classes of recombination events.

Plasmid maintenance is affected by recombination activity. Since plasmid copy number is determined through the control of the number of replication origins in the cell (19), plasmid oligomerization reduces the ratio of plasmid molecules to plasmid origins and, therefore, reduces also the number of plasmid molecules in the cell. This reduction may interfere with partition of plasmids to daughter cells, especially when unit copy-number plasmids, or high copy-number plasmids which lack a partition function, are involved.

The problems of oligomerization is overcome by the activity of specific recombination systems which resolve oligomers to monomeric products. Recombinogenic elements which are involved in site-specific recombination or activate recA-dependent intraplasmidic recombination have been described both in unit copy-number and in high copy-number plasmids (20,21,22).

THE MOLECULAR NATURE OF PLASMID RECOMBINATION

The Role of Strand Exchange

Two classes of genetic exchange can lead to the reconstruction of a functional gene by recombination of 2 genes mutated at different sites: one involves a double-strand exchange between the mutation sites, and the other a single-strand transfer, which is followed by branch migration across one or both mutations and processing of the heteroduplex intermediate to homoduplex by replication or mismatch repair (23). While the first mechanism contributes to recombination between distal markers, the second is involved mainly in recombination between close markers (24,25,26).

A system which facilitates the study of the molecular nature of plasmid recombination has been constructed (2,3). This system consists of 2 copies of the tet gene, each of which is mutated at a different restriction endonuclease site. The mutated genes are located on 2 compatible plasmids, for the analysis of intermolecular recombination (Fig. 1A), or, as a tandem repeat on one plasmid, for the analysis of intramolecular recombination (Fig. 1B). Recombination events are scored by determining the frequency of Tet^r recombinants, and the molecular structure of plasmid recombination products, which confer tetracycline-resistance, is determined by restriction endonuclease analysis. Mutations are induced in the tet gene by plasmid linearization with the appropriate restriction endonuclease, subsequent conversion of the single-strand ends to blunt ends by S1 nuclease digestion (deletions), or DNA polymerase 1-induced synthesis of the complementary strand (additions), and religation. Alternatively, molecular linkers or DNA sequences of various lengths are inserted at the restriction sites (2,3,4,5). In an interplasmidic recombination system, derivatives of pACYC184 (27) and pBR322 (28) are used as recombination substrates. These 2 plasmids share a sequence of 1.4 kb which is bound at one side by the EcoRI site on pBR322. Each of these plasmids confers antibiotic-resistance, in addition to the resistance to tetracycline; pACYC184 confers resistance to chloramphenicol, and pBR322, to ampicillin.

The molecular mechanism which yields tet^+ recombination products determines their structure. Structural analysis of these products can, therefore, contribute to the understanding of the recombination pathway (2,4,5). Examples of alternative interplasmidic recombination reactions in the BamHI-SalI interval of the tet gene, which yield distinct classes of recombination products, are presented in Fig. 2 and Fig. 3. Recombination of a pACYC184 derivative mutated at the SalI site, with a pBR322 derivative mutated at the BamHI site, can yield tet^+ products through a reciprocal double-strand exchange between 2 sites (Fig. 2), or by a process of single-strand exchange across one or both restriction sites and repair of the mismatched sequences (Fig. 3). The first possible pathway would

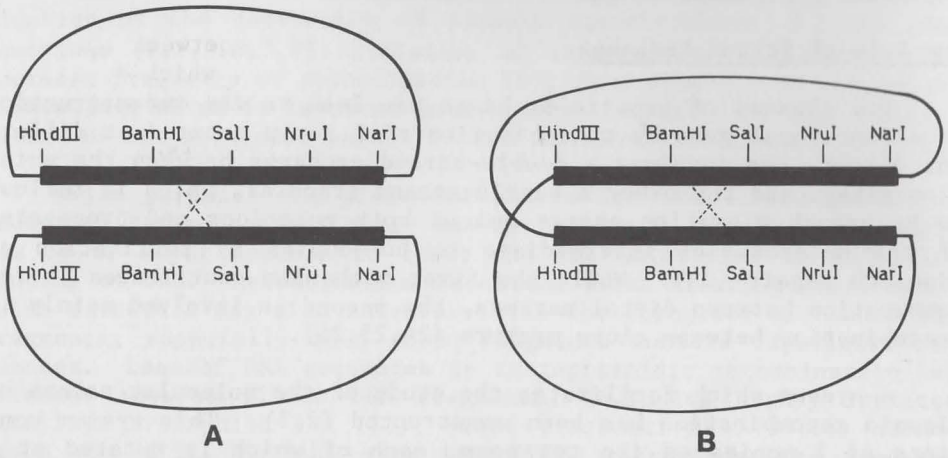


Fig. 1. Intermolecular and intramolecular recombination of the mutated tet gene. For intermolecular recombination, tet genes on 2 compatible plasmids (pBR322 and pACYC184) are mutated, each at a different restriction endonuclease site (A). In intramolecular recombination (B), both mutated genes are located as a tandem repeat on one plasmid. Recombination proficiencies are determined by scoring Tet^r cells (2,3). Recombination products are subjected to restriction endonuclease analysis (4,5).

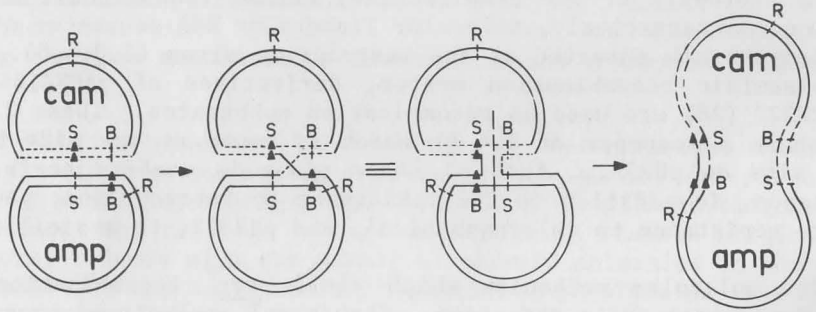


Fig. 2. The anticipated tet^+ interplasmidic recombination products of a reciprocal double-strand exchange between mutated BamHI and SalI restrictions endonuclease sites. A pBR322 derivative conferring ampicillin resistance (amp) and mutated at the BamHI site (B) was recombined with a pACYC184 derivative conferring chloramphenicol resistance (cam) and mutated at the SalI site (S), to yield a tet^+ amp^+ cam^+ heterodimer with one site for each restriction enconuclease.

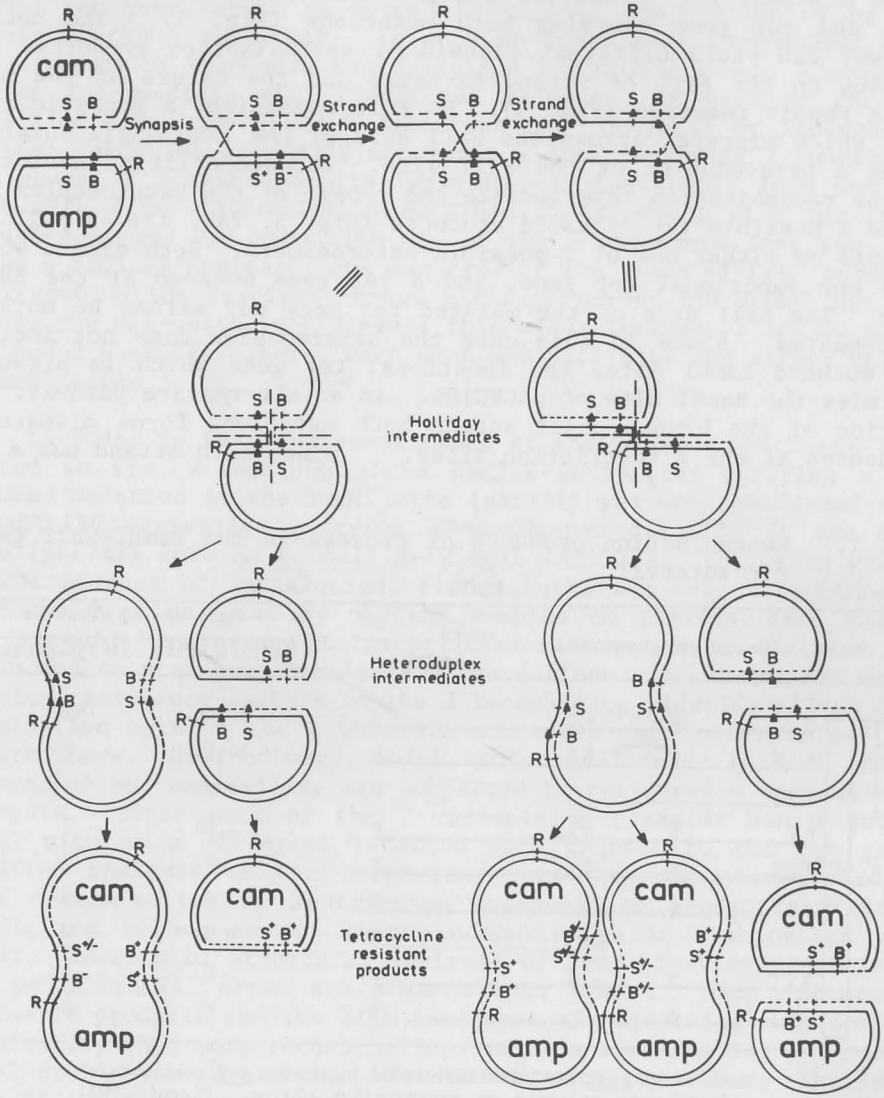


Fig. 3. Products of recombination reactions which involved heteroduplex formation and mismatch repair. In a recombination reaction between the substrates presented in Fig. 2, strand exchange across the SalI site and repair of the mismatched sequence can yield 3 possible tet^+ products; pACYC184 or one of 2 heterodimers (bottom left). Strand exchange across both mutation sites and independent repair of each mismatched sequence can yield 9 possible products (bottom right).

yield a single tet+ product: a heterodimer with one functional tet gene and one gene carrying both mutations (Fig. 2). The second pathway can yield different classes of recombination products, depending on the path of strand exchange and the nature of the mismatch repair reaction (Fig. 3). By this mechanism, a synaptic complex which migrates across the SalI site of the BamHI-SalI interval forms a heteroduplex at the SalI site. Endonucleolitic resolution of the recombination intermediate and repair of the heteroduplex can yield 3 possible tet+ plasmid products (Fig. 3, Tab. 1): a pACYC184 monomer or either one of 2 possible heterodimers. Both dimers would have one functional tet gene, and a tet gene mutated at the BamHI site. The SalI site of the mutated tet gene may either be mutated or repaired. Since in this case the heteroduplex does not include the mutated BamHI site, the functional tet gene which is produced includes the BamHI site of pACYC184. In an alternative pathway, migration of the branch point across both mutations forms mismatched sequences at the 2 restriction sites. Since each strand has a mutation at one of the sites and a wild allele at the other, a functional tet gene cannot be formed by plasmid replication or by co-repair of the 2 mutations. In such an event only independent mismatch repair at each site would yield a functional gene. A recombination intermediate with mismatched sequences at each of the restriction sites can yield 9 different tet+ products by independent repair: pACYC184 or pBR322 monomers, a dimer carrying 2 functional tet genes, 2 possible dimers carrying one functional gene and one gene with 2 mutations, and 4 possible dimers, each with one functional gene and one gene with one of the 2 mutations (see Tab. 1). Using a similar approach, one can predict the class of tet+ products if recombination involves strand exchange across the BamHI but not across the SalI site (not shown); this class includes a pBR322 monomer and 2 possible dimers, each with one SalI site and either one or 2 BamHI sites.

Tab. 1. Recombination products of crosses in the BamHI-SalI genetic interval.

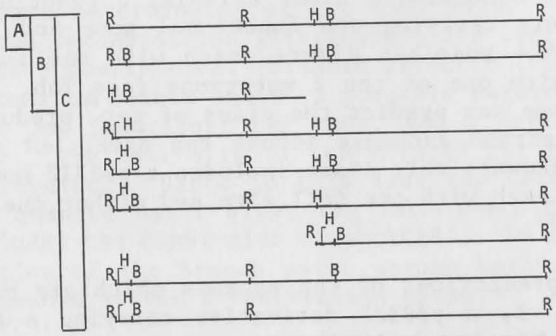
Recombining products	Recombining plasmids	
	pAL231xpAL242	pAL234xpAL246
A	21.1%	100%
B	30.3%	0
C	44.9%	0
	2.8%	0
	0	0
	0	0
	0.9%	0
	0	0
	0	0

Clones of E. coli K-12 (AB1157) cells harboring tet+ recombination products were selected on tetracycline plates. Plasmid DNA preparations of 2,000-4,000 Tet^r colonies were used to transform recA (DR100) cells, under conditions of limiting DNA concentration, and Tet^r transformants were analyzed for resistance to ampicillin and chloramphenicol (about 500 transformants for each cross). Plasmids of 59 Tet^r Amp^r Cam^r transformants of the cross pAL231xpAL242 and 67 of the cross pAL234xpAL246 were subjected to restriction endonuclease analysis. R, B, and S designate restriction endonuclease sites of EcoRI, BamBI, and SalI, respectively. The molecular structure which is indicated by box A is the anticipated product of a reciprocal double-strand exchange between the mutation sites (Fig. 2). Box B designates the anticipated products of a recombination reaction which involves a single-strand exchange across the SalI site and mismatch repair. The products which were marked by box C are anticipated from a recombination reaction which involves independent repair of mismatched sequences at both restriction sites (Fig. 3).

In order to test the predictions of the schemes which are presented in Fig. 2 and Fig. 3, a pBR322 derivative carrying a SI-induced deletion at the BamHI site (pAL231) was co-transformed with a pACYC184 derivative carrying a SI-induced deletion at the SalI site (pAL242) into an E. coli K-12 cell (AB1157). Following growth in the absence of antibiotics, clones harboring tet+ recombination products were selected by plating samples on tetracycline plates and plasmids conferring tetracycline-resistance were isolated and subjected to structural analysis. The differences between the antibiotics-resistance markers on the 2 recombining plasmids allows discrimination between the 2 possible monomeric tet+ products and the heterodimers. Heterodimers, which confer resistance to both chloramphenicol and ampicillin, are subjected to restriction endonuclease analysis. Since each of the 2 recombining plasmids has a unique EcoRI site at a different location with respect to the tet gene, detailed analysis of the heterodimer products is accomplished by size determination of restriction endonuclease products of SalI, BamHI, and either one of these endonucleases in combination with EcoRI. Results of structural analysis of recombination products of the pAL231xpAL242 cross are presented in Tab. 1. The presence of monomeric products and the high incidence of conversion rule out the possibility that most recombinations in this system involve a reciprocal double-strand exchange between the mutation sites. The location of the functional tet gene on most dimers, the observation that most tet+ monomers are pACYC184 and that the incidence of conversion at the SalI site is exceedingly higher than that at the BamHI site is consistent with the notion that most recombination events which yield tet+ products involve a single-strand exchange across the SalI site, but not across the BamHI site. The high incidence of gene conversion at the SalI site may suggest an asymmetric strand-exchange mechanism as proposed by Meselson and Radding (29).

Results of a recombination experiment in the HindIII-BamHI interval of the tet gene are presented in Tab. 2. A pBR322 derivative carrying an SI-induced deletion at the HindIII site (pAL230) was

Tab. 2. Recombination products of crosses in the HindIII-BamHI interval.

Recombining products	Recombining plasmids pAL230xpAL241
	36.7%
	46.4%
	16.2%
	0
	0
	0
	0.7%
	0
	0

Recombination products were analyzed as in Tab. 1. Except that HindIII, BamHI, and EcoRI restriction endonucleases were used. Forty-three Tet^r Cam^r Amp^r transformants were analyzed this way. R, B, and H indicate restriction sites of EcoRI, BamHI, and HindIII, respectively. Six hundred fifteen transformants were analyzed for resistance to ampicillin and chloramphenicol. Box A indicates the anticipated products of a reciprocal double-strand exchange between the mutation sites. Box B, the products of a recombination reaction which involves strand exchange across the BamHI site, and box C the products of a recombination reaction which involves strand exchange across both sites.

crossed with a pACYC184 derivative carrying an SI-induced deletion at the BamHI site (pAL241). Most tet$^+$ monomers obtained by this cross are also resistant to chloramphenicol, as occurs with the recombination substrate with the BamHI mutation. All dimers have a single HindIII site and most have 2 BamHI sites. The location of a single HindIII site in all products is the same as in pACYC184, 1.4 kb away from an EcoRI site.

Thus, in both crosses, most recombinations involved a conversion at the mutated restriction endonuclease site which is located at the distal position with respect to the HindIII site of the tet gene.

The results are consistent with a proposal that most recombination events which yield tet$^+$ recombinants are initiated outside the HindIII-SalI interval of the tet gene on the SalI side, and strand exchange is in the SalI → HindIII direction. This conclusion is also supported by the distribution of recombination products of crosses in the NruI-SalI and NruI-BamHI interval of the tet gene (data not shown).

The Effect of Nonhomologous Sequences on Strand Exchange

Do major nonhomologies block strand exchange? Studies of λ phage recombinations indicate that, in this system, major nonhomologies are included in the heteroduplex region but their resolution to functional products depends on DNA replication (Ref. 30, M. Lichten and M.S. Fox, in prep.). In an in vitro system, RecA-promoted strand exchange across major nonhomologies depends on ATP regeneration and on the presence of single-strand DNA binding protein (31). In the plasmid recombination system, major nonhomologies block strand exchange. A cross between a pBR322 derivative with a major insertion at the BamHI site (pAL234) and a pACYC184 derivative with a major insertion at the SalI site (pAL246) yields only one class of tet$^+$ recombination products -- heterodimers with one functional gene and one gene with both mutations (Tab. 1). The difference between the product of this cross and the product of the pAL231xpAL242 cross suggests that major nonhomologies are not included in a heteroduplex region, or, if included, are not repaired to yield a functional tet gene.

If, as suggested above, most recombination events in the pAL231xpAL242 cross are initiated outside the BamHI-SalI interval on the SalI side, and major nonhomologies block strand exchange, then a major insertion at the SalI site, but not at the BamHI site, should lower recombination frequency in this interval. Results which are presented in Tab. 3 are consistent with this prediction. Bracketing

Tab. 3. The effect of nonhomology on recombinant frequency in short genetic intervals.

Genetic interval	Recombining plasmids	Site of major non-homology	Recombinant frequency
BamHI-SalI	pAL231xpAL242	-	1×10^{-3}
BamHI-SalI	pAL234xpAL246	BamHI, SalI	4×10^{-5}
BamHI-SalI	pAL231xpAL246	SalI	9×10^{-5}
BamHI-SalI	pAL234xpAL242	BamHI	3.8×10^{-3}
HindIII-BamHI	pAL234xpAL240	BamHI	1.9×10^{-4}
HindIII-BamHI	pAL231xpAL240	-	2×10^{-3}

Recombinant frequencies were determined as described (2,5,8). The major nonhomology at the BamHI site is a 650 nucleotide sequence of λ phage CII region and at SalI site a 1,300 nucleotide sequence of Streptomyces griseus plasmid pSC1 (5).

the BamHI-SalI interval by inserting major nonhomologies at both restriction sites (pAL234xpAL246) leads to a major reduction in recombinant frequency. A major insertion at the SalI site (pAL231x pAL246) has a similar effect, but a major insertion at the BamHI site (pAL234xPAL242) does not lower recombination frequency. In fact, a major insertion at the BamHI site increases recombination frequency in the BamHI-SalI interval, possibly because of interference with strand exchange across the entire interval and, thereby, promoting endonucleolitic resolution of recombination intermediates within the interval. Heteroduplexes which are formed by such resolution can yield functional homoduplexes by replication or corepair. While a major insertion at the BamHI site does not decrease recombination proficiency at the BamHI-SalI interval, it decreases recombination proficiency in the HindIII-BamHI interval. Recombination frequencies of the pAL234xpAL240 are 10 times lower than recombination frequencies of the pAL231xpAL240 cross. This result also supports the proposal of initiation of recombination events at a site or a region outside the interval, on the SalI side, and a polar strand exchange in the SalI → HindIII direction. The nature of the hypothetical recombinogenic element which is involved in initiation of recombination events in this system, the mechanism of strand exchange and resolution of recombination intermediates, and the dependency of these processes on bacterial gene functions are under investigation.

THE DEPENDENCY OF PLASMID RECOMBINATION ON HOST FUNCTIONS

Two pathways are involved in postconjugational recombination of chromosomal DNA in *E. coli* K-12: the RecBC pathway which is responsible for most recombination events in the cell and the RecF pathway which is involved in about 1% of chromosome recombination (32). A third pathway, the RecE, can be activated in strains of *E. coli* which carry the *rac* prophage (33). Like the RecF pathway, the RecE pathway does not play a significant role in chromosomal recombination in wild-type cells. Mutations in the *recBrecC* genes, which inactivate the RecBC pathway and thereby reduce chromosomal recombination activity, can be suppressed by an *sbcA* or an *sbcB* mutation. The *sbcA* mutation activates the RecE pathway by depression of the synthesis of the *recE* gene product, exonuclease VIII (34). The *sbcB* mutation inactivates exonuclease I and thereby increases recombination activity via the RecF pathway (35). All 3 chromosomal recombination pathways have an absolute dependency on the *recA* gene product (32). The RecBC pathway also depends on the *recBrecC* gene product, exonuclease V. The RecE pathway depends on the *recE* gene product, exonuclease VIII, and on *recF* gene activity (36); the RecF pathway depends on functional *recF* and *recJ* activities (37) and on a *lexA*-controlled function which is coded by a gene located at 57.5 min on the *E. coli* chromosome (38).

Differences between the dependency of plasmid recombination and the dependency of chromosomal recombination on bacterial gene functions have been reported in studies involving recombination between 2 *Flac tra*$^-$ elements. Recombination in this system has a *recA* dependency, but is completely independent of the *recBrecC* gene product. On the other hand, this system has partial dependency on the *recF* function (1). Further characterization of the role of host gene functions in the process of plasmid recombination was accomplished by employing the interplasmidic and intraplasmidic recombination system which is described above (2,3,7,8).

These studies indicate several differences between the activities which are involved in postconjugational recombination and in plasmid recombination (Tab. 4). Like chromosomal recombination, plasmid recombination in wild-type cells depends on a functional *recA* gene product. However, while chromosomal recombination has an absolute dependency on the *recA* function, a residual plasmid recombination activity (1-4% of that in wild type cells) is observed in *recA* mutants (2,3). The dependency of intraplasmidic recombination on the *recA* function is completely alleviated when recombination

Tab. 4. The dependency of plasmid recombination on bacterial gene functions.

Bacterial relevant genotype (representative strain)	Relative recombinant frequency		
	Intraplasmidic	Interplasmidic	Postconjugational
rec$^+$ (AB1157)	1.0	1.0	1.0
recA (JC2926)	1.5×10^{-2}	2.7×10^{-2}	10^{-5}
recBrecC (JC5519)	1.0	1.0	10^{-2}
recF (JC9239)	2.6×10^{-2}	2.7×10^{-2}	1.0
sbcB (JC11451)	1.0	1.0	1.0
recBsbcA (JC5183)	32	4.8	0.5
RecArecBsbcA (DR107)	33	0.13	10^{-5}

Relative recombinant frequencies are determined in relation to frequencies in *rec*$^+$ (AB1157) cells. Relative plasmid recombinant frequencies are from Ref. 2, 8, and postconjugational recombination frequencies are of Ref. 32, 34, 36, 37. Recombinant frequencies of *recBsbcB* strains are not determined due to the instability of plasmids in this genetic background.

proceeds via the RecE pathway. Intraplasmidic recombination proficiency in recArecBsbcA cells is similar to that in recBsbcA cells. In both genetic backgrounds this proficiency is significantly higher than that in wild-type cells (2,3).

Interplasmidic recombination via the RecE pathway is partially dependent on the recA activity. Interplasmidic recombination proficiencies in recArecBsbcA cells is about 40-fold lower than that in recBsbcA (2).

RecA-independent recombination via the RecE pathway has also been observed with replicating λ DNA molecules as a substrate (39). It is conceivable that in both systems DNA replication intermediates serve as substrates for recA-independent recombination reaction. The activity which suppresses recA deficiency in the recBsbcA genetic background has not yet been characterized. An attractive possibility is the functioning of an activity, similar to that of λ phage β protein (40), which is associated with exonuclease VIII or with another prophage rac product.

Plasmid recombination in wild-type cells does not depend on the recBrecC gene product. It does however depend on the recF gene activity (7,8). Unlike chromosomal recombination, recombination of plasmids via a recF-dependent pathway is not enhanced by inactivation of exonuclease I by a sbcB mutation (8).

The differences between postconjugational chromosomal recombination and plasmid recombination in the degree of the dependency of these processes on recBrecC and recF functions suggest that the role of different gene products in recombination is determined by the molecular structure of the DNA substrate. It is possible that in vivo, like in vitro (41,42), circular DNA molecules do not serve as substrates for the recBrecC gene product, exonuclease V. Therefore, recombination of plasmids and other circular molecules depends on another enzymatic activity, possibly on the recF gene product. This may be related to the proposal that postconjugational recombination via the recF pathway involves circular intermediates (43). Understanding the nature of the enzymatic activity associated with the recF gene product and with the product of other genes which are involved in recombination via the RecF pathway will contribute to the investigation of the molecular nature of plasmid recombination.

REFERENCES

1. Willetts, N.S. (1975) Recombination and the Escherichia coli K-12 sex factor F. J. Bact. 121:36-43.
2. Laban, A., and A. Cohen (1981) Interplasmidic and intraplasmidic recombination in Escherichia coli K-12. Mol. Gen. Genet. 184:200-207.

3. Fishel, R.A., A.A. James, and R. Kolodner (1981) RecA independent general recombination of plasmids. Nature 294:184-186.
4. Doherty, M.J., P.T. Morrison, and R. Kolodner (1983) Genetic recombination of bacterial plasmid DNA. Physical and genetic analysis of the products of plasmid recombination in Escherichia coli. J. Mol. Biol. 167:539-560.
5. Laban, A., Z. Silberstein, and A. Cohen (1984) The effect of non-homologous DNA sequences on interplasmidic recombination. Genetics (in press).
6. Potter, H., and D. Dressler (1976) On the mechanism of genetic recombination: Electron microscopic observation of recombination intermediates. Proc. Natl. Acad. Sci., USA 73:3000-3004.
7. James, A.A., P.T. Morrison, and R. Kolodner (1982) Genetic recombination of bacterial plasmid DNA. Analysis of the effect of recombination-deficient mutations on plasmid recombination. J. Mol. Biol. 160:411-430.
8. Cohen, A., and A. Laban (1983) Plasmidic recombination in Escherichia coli K-12: The role of recF gene function. Mol. Gen. Genet. 189:471-474.
9. James, A.A., P.T. Morrison, and R. Kolodner (1982) Isolation of genetic elements that increase frequencies of plasmid recombinants. Nature 303:256-259.
10. Jones, I.M., S.B. Primrose, and S.D. Ehrlich (1982) Recombination between short direct repeats in a recA host. Mol. Gen. Genet. 188:486-489.
11. Albertini, A.M., M. Hofer, M.P. Calos, and J. Miller (1982) On the formation of spontaneous deletions: The importance of short sequence homologies in the generation of large deletions. Cell 29:319-328.
12. Brutlag, D., K. Fry, T. Nelson, and P. Hung (1977) Synthesis of hybrid bacterial plasmids containing highly repeated satellite DNA. Cell 10:509-519.
13. Cramer, J.H., F.W. Farrelly, J.T. Barnitz, and R.H. Rownd (1977) Construction and restriction endonuclease mapping of hybrid plasmids containg S. cerevisiae ribosomal DNA. Mol. Gen. Genet. 151:229-244.
14. Cohen, A., D. Ram., H.O. Halverson, and P.L. Wensink (1978) Deletions within E. coli plasmids carrying yeast rDNA. Gene 3:135-147.
15. Perricauset, M., A. Fritsch, U. Pattersson, L. Philipson, and P. Thiollais (1977) Excision and recombination of adenovirus DNA fragments in Escherichia coli. Science 196:208-210.
16. Case, S.T. (1982) Selective deletion of large segments of Balbiani ring DNA during molecular cloning. Gene 20:169-176.
17. Keshet, E., and Y. Shaul (1981) Terminal direct repeats in a retrovirus-like repeated mouse gene family. Nature 289:83-85.
18. McClements, W.L., L.W. Enquist, M. Oskarsson, M. Sullivan, and G.F. Vande Woude (1980) Frequent site-specific deletion of Coliphage λ murine sarcoma virus recombinants and its use in the identification of a retrovirus integration site. J. Virol. 35:488-497.

19. Prichard, R.H., and N.B. Grover (1981) Control of Plasmid Replication and its Relationship to Incompatibility. In Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, S.B. Levi, R.C. Clowes, and E.L. Koenig, eds. Plenum Press, New York, pp. 271-278.
20. Austin, S., M. Ziese, and N. Sternberg (1981) A novel role for site-specific recombination in maintenance of bacterial replicons. Cell 25:729-736.
21. Hakkaart, M.J.J., E. Veltkamp, and H.J.J. Nijkamp (1982) Maintenance of the bacteriocinogenic plasmid Clo DF13 in Escherichia coli cells. II. Specific recombination functions involved in plasmid maintenance. Mol. Gen. Genet. 188:338-344.
22. Hakkaart, M.J.J., P.J.M. van den Elzen, E. Veltkamp, and H.J.J. Nijkamp (1984) Maintenance of multicopy plasmid Clo DF13 in E. coli cells: Evidence for site-specific recombination at parB. Cell 36:203-209.
23. Holliday, R. (1964) A mechanism for gene conversion in fungi. Genet. Res. 5:282-304.
24. Amati, P., and M. Meselson (1965) Localized negative interference in bacteriolophage λ. Genetics 51:369-379.
25. Meselson, M. (1967) The Molecular Basis of Genetic Recombination. In Heritage from Mendel, R.A. Brink, ed. The University of Wisconsin Press, Madison, pp. 81-104.
26. Fox, M.S. (1978) Some features of genetic recombination in procaryotes. Ann. Rev. Genet. 12:47-68.
27. Chang, A.C.Y., and S.N. Cohen (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bact. 134:1141-1156.
28. Bolivar, F., R.L. Rodriguez, P.J. Green, M.C. Betlach, H.L. Heyneker, H.W. Boyer, J.H. Crosa, and S. Falkow (1977) Construction and characterization of new cloning vehicles. II. A multi-purpose cloning system. Gene 2:95-113.
29. Meselson, M.S., and C.M. Radding (1975) A general model for genetic recombination. Proc. Natl. Acad. Sci., USA 72:358-361.
30. Lichten, M., and M.S. Fox (1983) Effect of non-homology on bacteriophage lambda recombination. Genetics 103:5-22.
31. Bianchi, M.E., C. DasGupta, and C.M. Radding (1983) Insertions, deletions and mismatches in heteroduplex DNA made by RecA protein. Cell 34:931-939.
32. Clark, A.J. (1973) Recombination deficient mutants of E. coli and other bacteria. Ann. Rev. Genet. 7:67-86.
33. Kaiser, K., and N.E. Murray (1979) Physical characterization of the "rac prophage." Mol. Gen. Genet. 175:159-174.
34. Barbour, S.D., H. Nagaishi, A. Templin, and A.J. Clark (1970) Biochemical and genetic studies of recombination proficiency in Escherichia coli. II. Rec$^+$ revertants caused by indirect suppression of Rec mutations. Proc. Natl. Acad. Sci., USA 67:128-135.
35. Kushner, S.R., H. Nagaishi, and A.J. Clark (1972) Indirect suppression of recB and recC mutations by exonuclease I deficiency. Proc. Natl. Acad. Sci., USA 69:1366-1370.

36. Gillen, J.R., D.K. Willis, and A.J. Clark (1981) Genetic analysis of the RecE pathway of genetic recombination in Escherichia coli K-12. J. Bact. 145:521-532.
37. Horii, Z.I., and A.J. Clark (1973) Genetic analysis of the RecF pathway of genetic recombination in Echerichia coli K-12. Isolation and characterization of mutants. J. Mol. Biol. 80:327-344.
38. Lloyd, R.G., S.M. Picksley, and C. Prescott (1983) Inducible expression of a gene specific to the RecF pathway. Mol. Gen. Genet. 190:162-167.
39. Gillen, J., and A.J. Clark (1974) The recF pathway of bacterial recombination. In Mechanism in Recombination, R.F. Grell, ed. Plenum Press, New York, pp. 123-136.
40. Kmiec, E., and W.K. Hollman (1981) β protein of bacteriophage λ promotes renaturation of DNA. J. Biol. Chem. 256:12636-12639.
41. Karu, A.E., V. Mackay, P.S. Goldmark, and S. Linn (1973) The recBC deoxyribonuclease of Escherichia coli K-12. Substrate specificity and reaction intermediates. J. Biol. Chem. 248:4874-4884.
42. Taylor, A., and G.R. Smith (1980) Unwinding and rewinding of DNA by the RecBC enzyme. Cell 22:447-457.
43. Bresler, S.E., I.Y. Goryshin, and V.A. Lanzov (1981) The process of general recombination in Escherichia coli: Structure of intermediate products. Mol. Gen. Genet. 183:139-143.

THE ORIGIN OF PLASMID DNA TRANSFER DURING BACTERIAL CONJUGATION

Donald G. Guiney, Gary Chikami, Cornelia Deiss,
and Emanuel Yakobson

Department of Medicine H811F
University of California Medical Center
225 Dickinson Street
San Diego, California 92103

INTRODUCTION

Bacterial conjugation is the process of gene transfer requiring cell-to-cell contact. Such mating systems are widespread in bacteria and have been described in many gram-positive and gram-negative organisms (1-3). However, detailed studies on the mechanism of DNA transfer during conjugation have been carried out only in gram-negative bacteria (4). The conjugation systems studied in gram-negatives are encoded by plasmids, and mediate the transfer of plasmid and, occasionally, chromosomal DNA from the donor to the recipient cell. Although transformation and transduction of plasmids occur, conjugation appears to be the major mechanism of plasmid DNA transfer in nature. In addition, conjugation is the only means of genetic exchange between different groups of bacteria, since transformation and transduction are generally limited to exchange between closely related members of the same genus or species. The promiscuous gene transfer mediated by broad host-range plasmids means that the gene pool available to bacteria is immensely larger than if genetic exchange was restricted by species barriers. Therefore, broad host-range conjugation enables bacteria to draw upon a very large genetic reservoir when subjected to evolutionary pressures. The most dramatic clinical example of this exceptional adaptability is the development and spread of antibiotic resistance in pathogenic bacteria.

Current knowledge of the molecular mechanisms of plasmid DNA transfer during conjugation is based heavily on studies of the F plasmid in Escherichia coli (reviewed in Ref. 1, 2, and 4). The general features of F plasmid transfer are shown in Fig. 1. The F

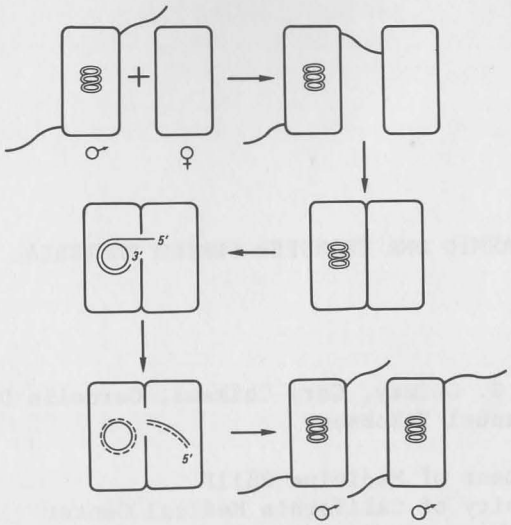


Fig. 1. Scheme of bacterial conjugation, based on the F plasmid of E. coli. For simplicity, the chromosomal DNA is not shown. Plasmid DNA synthesized in the donor and recipient during conjugal DNA replication is shown by the dashed lines.

sex pili are required to establish early mating contact between the donor and recipient cells (5). The donor and recipient then form stable mating pairs or aggregates with extensive wall-to-wall contacts (6). A mating bridge is formed, and a mating signal triggers conjugal DNA replication and transfer in the donor cell. A single specific strand is transferred to the recipient with the 5' end leading (7,8). Conjugal DNA synthesis in the donor replaces the transferred strand, while a complement to this strand is made in the recipient. The transferred strand is recircularized and ligated in the recipient to form the intact plasmid. The extent to which this scheme of F plasmid transfer can be generalized to other plasmids is not known. Single strand-specific transfer with 5' orientation has not been demonstrated for other plasmids. Nevertheless, the model shown in Fig. 1 forms the basis for studies of other plasmid conjugation systems.

The origin of transfer (oriT) is the region of particular interest in studying the molecular mechanism of DNA transfer. It is at oriT that the major events of conjugative DNA metabolism occur: nicking of the transferred strand, conduction from donor to recipient, initiation of conjugal DNA synthesis, and recircularization in the recipient. Because of this central role in conjugation, the oriT region has received detailed study.

PLASMID DNA-PROTEIN RELAXATION COMPLEXES

Properties of Relaxation Complexes

The discovery of plasmid DNA-protein relaxation complexes has provided a key to the mechanism of initiation of DNA transfer at oriT. Many bacterial plasmids, including resistance plasmids and colicinogenic factors, can be isolated from gently lysed cells in the form of a superhelical DNA-protein complex, which can be induced in vitro to nick the DNA and convert the plasmid to the open circular form (Fig. 2) (9,10). In the case of the ColE1 plasmid for E. coli, the nick occurs at a specific site in the heavy strand, and a 60,000 d protein remains covalently attached to the 5' end of the DNA (11-13). These results led to the proposal that the relaxation complex initiates plasmid transfer in vivo by nicking at the origin of transfer, mediates movement of the 5'end of the DNA into the recipient, and promotes recircularlization and ligation of the plasmid (13,14). This hypothesis was substantiated by Sherratt and coworkers who showed that the ColE1 relaxation complex is required for mobilization and that the oriT and relaxation nick sites coincide (14,15).

Triggering of Relaxation Complex Nicking

The in vivo signal that triggers relaxation complex nicking is unknown. The initial in vitro studies found that relaxation was induced by agents which disrupted protein structure: pronase, ionic

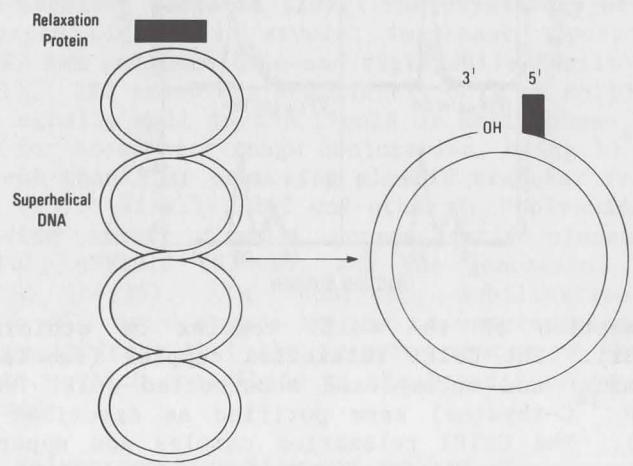


Fig. 2. Reaction mediated by plasmid relaxation complexes. The superhelical DNA is tightly bound to one or more relaxation proteins. Relaxation induces a strand- and site-specific nick in the DNA. In the open circular product, the 3' end of the nicked strand is free, but the 5' end is covalently linked to one of the relaxation proteins (13).

detergents such as sodium dodecyl sulfate, and heat (9). We have found that changes in the DNA structure also trigger the nicking event. Ethidium bromide, which unwinds the DNA helix, is a very efficient inducer of relaxation. Furthermore, as shown in Fig. 3, the nicking event occurs at an ethidium bromide concentration which just removes all the negative superhelical turns in the native plasmid DNA. In this experiment, the supercoiled plasmid DNA-protein complex is sedimented through sucrose gradients containing different concentrations of ethidium bromide. As ethidium bromide binds to superhelical DNA, it removes the negative superhelical turns and decreases the sedimentation rate (16). At an ethidium bromide concentration of about 2 μg/ml, there are no superhelical turns remaining, and the DNA sediments as an open circle (see Panel C, Fig. 3). At higher concentrations, positive superhelical turns are introduced, and the sedimentation rate increases again. Figure 3 shows that ethidium bromide concentrations above 2 μg/ml induce nicking by the relaxation complex, so that most of the plasmid DNA continues to sediment as open circles, even in higher concentrations of ethidium (see Panels D, E, F of Fig. 3). These results show that DNA unwinding triggers the nicking event, and suggest that physiological processes such as DNA replication and transcription, which also unwind DNA, may trigger nicking in vivo. There may be a continuous equilibrium in the cell between nicked and covalently closed forms of the relaxation complex. For some plasmids, nicking may not be triggered by a specific mating signal, but rather this continuous nicking process could supply open circular plasmid molecules ready for transfer. Such a continuous nicking and ligation process would explain the results of Everett and Willetts, who found that phages containing the F transfer origin were nicked even in cells containing F mutants unable to make sex pili (17). Nicking and ligation by the ColE1 relaxation complex during replication would also account for the properties of a temperature-sensitive relaxation complex mutation isolated by Collins and Helinski (18). The relaxation complex of this plasmid was unstable at elevated temperatures. The mutant plasmid was also temperature sensitive for replication, yet other studies showed that the relaxation complex is not required for replication (14,15,19). However, if the nicking/ligation function was disrupted at the nonpermissive temperature, this plasmid could be "stuck" in the open circular state and be unable to complete replication.

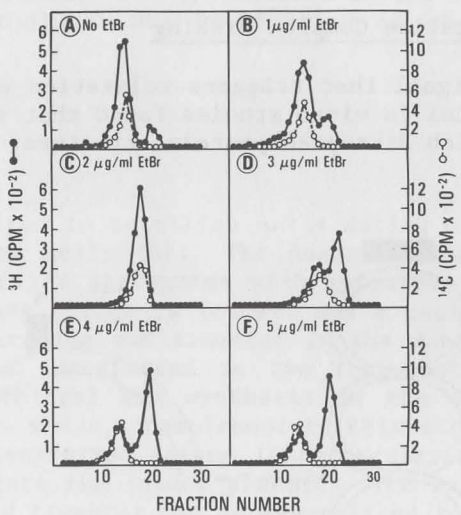


Fig. 3. Relaxation of the ColE1 complex by ethidium bromide (EtBr). The ColE1 relaxation complex (labeled with ^{3}H-thymine) and uncomplexed supercoiled ColE1 DNA (labeled with ^{14}C-thymine) were purified as described previously (13). The ColE1 relaxation complex and supercoiled DNA marker were sedimented through 5-20% sucrose gradients containing EtBr at the indicated concentrations. Methods for centrifugation, fractionation, and radioisotope counting are described in Ref. 13. This experiment was also done using ^{14}C-labeled ØX174 single-strand DNA as a marker to show that the concentration of EtBr needed to remove the negative superhelical turns is 2 μg/ml.

THE TRANSFER ORIGIN OF RK2

The conjugation system of IncP plasmid RK2 is of particular interest because of the broad host range properties of this plasmid. The RK2 transfer system is particularly well adapted to overcome species barriers and can mediate gene transfer between a wide variety of gram-negative bacteria (20). The physiology of the RK2 conjugation system differs in several important aspects from the F plasmid. RK2 sex pili are thin and rigid while F pili are thick and flexible (21). RK2 transfers efficiently only on solid medium while F transfers equally well in the liquid or solid phase (22). RK2 is well suited for broad host range conjugation, being 10^4 to 10^5 times more efficient than F at promoting plasmid transfer from E. coli to Pseudomonas (23). Finally, RK2 and other IncP plasmids are able to mobilize a wide variety of small, nonconjugative plasmids, including ColE1, pSC101, RSF1010 (R300B), and the gonococcal penicillinase plasmid pWD2 (24,25). In contrast, mobilization of pSC101, RSF1010, and pWD2 by F occurs at low to undetectable frequencies. These studies indicate that the transfer system of RK2 has special characteristics which contribute to the broad host range properties of the plasmid.

Mapping the Relaxation Nick Site and oriT of RK2

Figure 4 shows a map of RK2 with the location of the relaxation nick site (rlx) and the transfer origin (oriT) which coincide at the 48 kb coordinate. The rlx site was located by sizing the single-stranded DNA after nicking, restriction enzyme digestion, and alkaline denaturation (26). oriT was defined by genetic criteria:

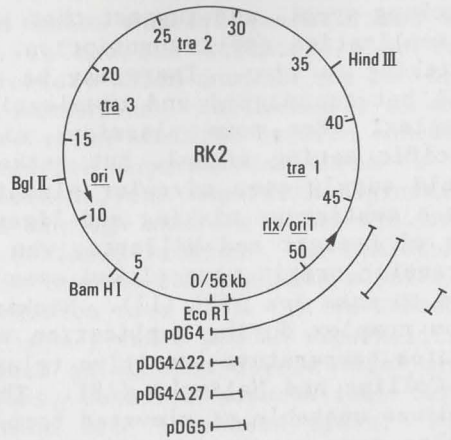


Fig. 4. Map of RK2 (taken from Ref. 27). tra 1,2, and 3 are the 3 transfer regions defined by Barth (34,35) on the basis of Tn7 insertions. rlx is the relaxation nick site (26) and oriT is the transfer origin (27). The RK2 DNA cloned into the pDGV1 vector in the derivatives pDG4, pDG4Δ22, pDG4Δ27, and pDG5 is shown by the solid bars.

1) the oriT site is required in cis for transfer, 2) oriT confers high frequency, recA independent mobilization when the conjugation system is supplied by a helper plasmid in trans, and 3) cointegrate formation is not involved in mobilization (27). The functional oriT region was subcloned as a 760 bp HaeII fragment on pDG5 (Fig. 5).

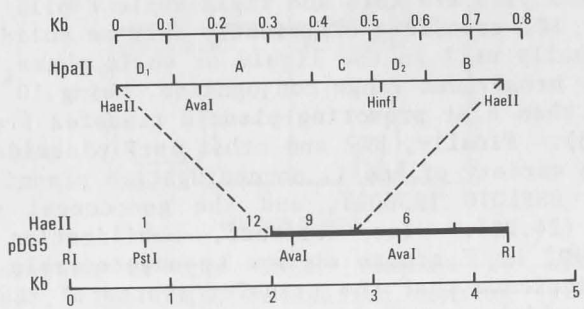


Fig. 5. Map of pDG5. The RK2 DNA present in pDG5 is shown by the heavy line. pDG5 was generated in several steps by partial HaeII digests of pDG4. The HaeII numbers refer to fragments from pDG4 that are present in pDG5 (see Fig. 4). Fragment 9 is a 760 bp sequence spanning oriT, and fragment 6 is from the region just counterclockwise from the EcoRI site of RK2. HpaII digestion of fragment 9 yields 5 subfragments labeled A,B, C, D_1, and D_2 according to decreasing size.

This HaeII fragment contains 4 HpaII sites producing 5 subfragments, designated A, B, C, D_1, and D_2 according to size. The D_2 and B HpaII fragments were cloned into the EcoRI/ClaI region of the vector pDGV1. As shown in Tab. 1, the fully functional oriT is contained in the D_2B region, since pDG16 is transferred nearly as efficiently as pDG5 by the pRK231 helper plasmid. The D_2 fragment alone functions as the transfer origin, but the transfer frequency of pEYDG59 containing D_2 is reduced to about 1% of the frequency of pDG16. Since pDG17 (containing B alone) is not mobilized, the B region by itself is not a transfer origin, but B is required for full function of the oriT activity contained in D_2.

The DNA sequence of the 112 bp HpaII D_2 fragment has been determined and is shown in Fig. 6 (27). The most striking feature of the region is a 19 bp inverted repeat in the right-hand half of the sequence. This inverted repeat could form a hairpin or cruciform structure, as shown in Fig. 7. We have postulated that this inverted repeat may be the binding site for the RK2 relaxation complex. This suggestion is strengthened by our subsequent finding that deletion of 22 bp from the right end of the sequence (forming plasmid pDG11, see Fig. 6) completely abolishes oriT activity (see transfer data in Tab. 1). The deletion in pDG11 removes the outside 10 bp from the right-hand inverted repeat, and indicates that the intact inverted repeat is required for oriT activity. This deletion also

Tab. 1. Mobilization of oriT containing plasmids.

Plasmid[a]	oriT HpaII Fragments[b]	Transfer Frequency[c]
pDG5	D_1ACD_2B	1.4
pDGV1	none	<0.00001
pDG16	D_2B	0.7
pDG17	B	<0.00001
pEYDG59	D_2	0.003
pDG11	D_2 deletion	<0.00001

[a] All plasmids were transformed into E. coli HB101 containing the RK2 helper plasmid pRK231 (tra$^+$, Tc^R, Km^R).

[b] The HpaII D_2,B, and D_2B fragments were cloned into the EcoRI/ClaI sites of the vector pDGV1. All fragments are in the same orientation with respect to the vector.

[c] The donors (HB101 containing pRK231 and the indicated plasmid) were mated with the HB101 nal^R recipient on filters as described previously (27). Transfer frequency is the number of transconjugants containing the mobilized plasmid divided by the number of transconjugants containing pRK231.

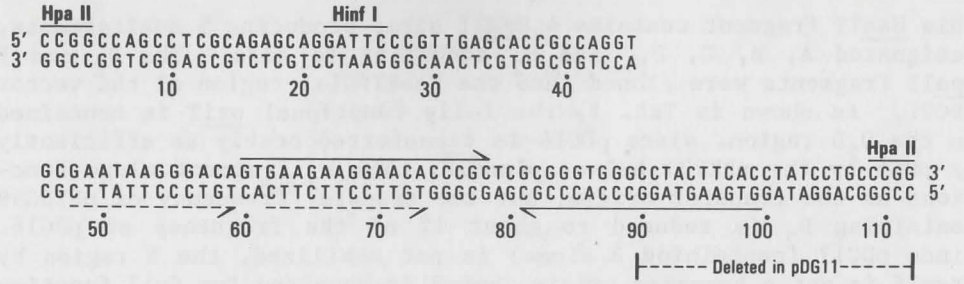


Fig. 6. Nucleotide sequence of HpaII D_2 fragment containing oriT. The inverted repeat is shown by solid arrows, and the direct repeat is seen with dashed arrows. The 22 bp deleted in pDG11 is also shown, and extends from a HaeIII site at base pair 90 to the end of the sequence.

shows that the two direct repeats do not function as a transfer origin.

Homology in the Transfer Origins of IncP Plasmids

RK2 is similar or identical to RP1, RP4, R18, and R68 (28). All these plasmids were isolated in Birmingham, England, and are epidemiologically related (29). A number of other IncP plasmids have been isolated from around the world and carry different drug resistance markers. We have examined the DNA homology between the RK2 oriT region and 9 other IncP plasmids isolated from different geographic locations (30). Figure 8 shows a representative Southern hybridization between the 760-bp HaeII oriT fragment of RK2 and a HaeII digest of 5 other IncP plasmids. R996 and pUZ8 contain strongly hybridizing HaeII fragments of identical size to the HaeII oriT fragment of RK2, while for R772, R906, and R751, the homologous HaeII fragment is smaller and the hybridization is much weaker. The other IncP plasmids tested, R702, R839, R938, and R1033, all had homologous HaeII fragments identical to RK2. We have extended these homology studies to examine 3 other important regions of RK2: 1) the primase gene, 2) the trfA region, which produces a trans-acting product required for replication, and 3) the oriV region. For each of these regions, we find that 6 IncP plasmids are identical to RK2: R702, R839, R938, R995, pU28 and R1033. The 3 other IncP plasmids, R751, R906, and R772, are similar to each other but quite different in the pattern of hybridization from RK2. Based on these hybridization studies, we have designated the RK2-like plasmids as IncPα and R751-like plasmids as IncPβ (30,31). The recent heteroduplex results of Villarroel et al. show that the IncPα plasmids all share a common core structure (32). The homology studies indicate that the IncP plasmids diverged some time ago into 2 distinct evolutionary groups that continue to share incompatibility and broad host-range properties.

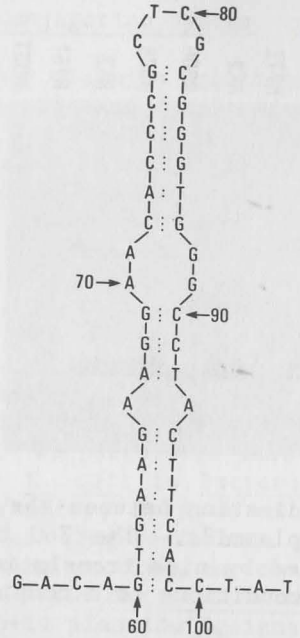


Fig. 7. Potential hairpin structure involving the inverted repeats in oriT. There are 3 bp mismatches, and the first base pair of the loop probably does not form due to steric hindrance. The free energy of formation from single-stranded DNA is -10.8 kcal mol^{-1} (calculated by the method of Tinoco et al., 36). The deletion in pDG11 reduces the free energy of formation to -6.0kcal mol^{-1}.

Specificity of the RK2 oriT Region

Since the homology data indicated substantial differences between the oriT regions of IncPα and IncPβ plasmids, we tested the functional relationships between the transfer systems of these plasmids. Table 2 shows the mobilization frequency of plasmids containing the RK2 oriT region using R702 (IncPα) and R751 (IncPβ) as the tra^+ helper plasmids. R702 is able to transfer pDG5 (760 bp oriT) and pEYDG59 (112 bp oriT) as efficiently as RK2. However, R751 was not able to mobilize either pDG5 or pEYDG59, but could transfer pDG4Δ22 and pDG4 as efficiently as RK2 (see Fig. 4 for maps of pDG4Δ22 and pDG4). Similar results were obtained with other IncPα and IncPβ plasmids. These studies show that the IncPβ transfer system cannot utilize the oriT of RK2, probably because the IncPβ relaxation proteins are unable to recognize the IncPα oriT sequence. However, since pDG4 forms a functional RK2 relaxation complex, this can be recognized by the rest of the IncPβ conjugation system and efficient mobilization occurs. Since pDG4Δ22 is also transferred by R751, the RK2-specific gene product which interacts with oriT is

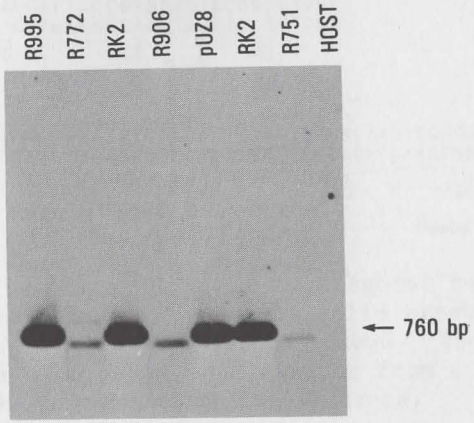


Fig. 8. Southern hybridization between the oriT region of RK2 and 5 other IncP plasmids. The 760 bp HaeII fragment 9 of pDG5 was labeled by nick translation and hybridized under low strigency conditions to a complete HaeII digest of the IncP plasmids.

contained within a 3 kb region surrounding oriT (see Fig. 4). These studies show that the functional specificity of the IncP conjugation system is at the level of recognition of the oriT sequence by the relaxation complex. Once this binding is accomplished, transfer can occur by either the IncPα or IncPβ systems. However, the interaction of conjugation systems with the RK2 relaxation complex is specific for the IncP group, since pDG4 cannot be mobilized by Flac or R64drd11.

Tab. 2. Mobilization of the RK2 oriT region by IncPα and IncPβ plasmids.

Transfer Plasmid[a]	Mobilization of RK2 oriT Plasmid[b]			
	pEYDG59	pDG5	pDG4 Δ22	pDG4
R702 (IncPα)	1.3	1.0	—	—
R751 (IncPβ)	0.001	0.0001	0.1	15

[a]The RK2 oriT plasmids were transformed into E. coli HB101 containing either R702 or R751.

[b]Matings were done as described in Table 1. Mobilization is expressed as the ratio: transfer of the RK2 oriT plasmid by the indicated IncP plasmid divided by transfer of the RK2 oriT plasmid by pRK231.

Host Range of the RK2 Conjugation System

RK2 and related IncP plasmids can transfer to and be maintained in a wide variety of aerobic and facultative gram-negative bacteria. This broad host-range requires a conjugation system capable of transfer between unrelated bacteria, and a replication system able to maintain the plasmid in the new host. In a direct comparison of the RK2 and F plasmid transfer systems, RK2 was 10^4 to 10^5 times more efficient than F at transfers between E. coli and Pseudomonas aeruginosa (23). Despite the extensive host range of the IncP plasmids, we have been unable to transfer RK2 into the gram-negative anaerobe, Bacteroides. These organisms are the most numerous bacteria in the human colon, and thus are in close contact with E. coli. However, systematic attempts to transfer both narrow and broad host-range plasmids from E. coli to Bacteroides fragilis have been unsuccessful (20). We postulated that this barrier to successful gene transfer from E. coli to Bacteroides could be due to 1) lack of plasmid DNA transfer, 2) lack of RK2 replication in Bacteroides, or 3) lack of drug-resistance gene expression in Bacteroides. To examine this problem, we constructed a novel shuttle vector consisting of pDG5 and the Bacteroides clindamycin resistance plasmid pCP1. This hybrid plasmid, designated pDP1 (Fig. 9) replicates in both E. coli and B. fragilis, and contains the RK2 oriT sequence (33). As seen in Tab. 3, pDP1 can be transferred into B.

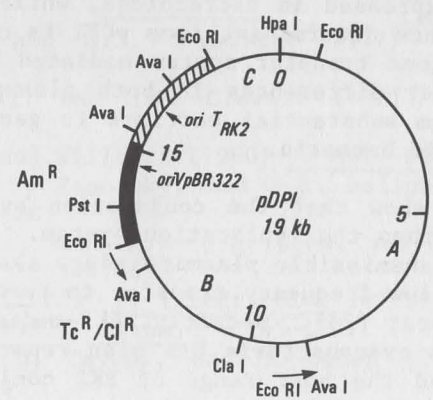


Fig. 9. Map of pDP1, showing pDG5 (thick line) linked to the Bacteroides plasmid pCP1 (thin line). The region of pBR322 containing oriV and ampicillin resistance (Am^R) is shown by the thick solid line, and the RK2 DNA containing oriT is seen by the thick hatched line. The three EcoRI fragments of pCP1 are labeled A, B, and C. The arrows show the location of the direct repeats of pCP1. The EcoRI B fragment of pCP1 encodes tetracycline resistance (Tc^R) in E. coli and clindamycin resistance (Cl^R) in Bacteroides (37).

Tab. 3. Transfer of pDP1 into Bacteroides fragilis.

Plasmids in E. coli Donor[a]	Transfer Frequency to B. fragilis[b] pDP1	pRK231
pDP1	$<10^{-8}$	—
pDP1+pRK231	3×10^{-6}	$<10^{-8}$

[a] E. coli donor was JA221 leuB, recA, hsdR$^-$, hsdM$^+$.

[b] The B. fragilis recipient was strain 638 rif^R. Transfers were performed by a plate mating technique (33). Transfer frequency is the number of transconjugants containing the selected plasmid divided by the total number of B. fragilis recipients present at the end of the overnight matings. pDP1 transfer was selected with 5 ug/ml of clindamycin and pRK231 was selected with 1 ug/ml of tetracycline.

fragilis by the RK2 helper plasmid, pRK231. However, pRK231 DNA was never found in the B. fragilis transconjugants selected for pDP1 transfer. Since pRK231 is almost always cotransferred in E. coli matings, we interpret these results to indicate that pRK231 cannot be maintained in Bacteroides. Furthermore, the TEM β-lactamase gene from pDG5 is not expressed in Bacteroides, while the erythromycin-clindamycim resistance determinant from pCP1 is not expressed in E. coli. This novel gene transfer system mediated by RK2 has allowed us to determine that differences in both plasmid replication and gene expression form substantial barriers to gene exchange between aerobic and anaerobic bacteria.

These studies show that the conjugation system of RK2 has a broader host-range than the replication system. This may be a general property of transmissible plasmids since the E. coli F plasmid is able to mediate low frequency transfer to Pseudomonas but cannot replicate in that host (23). Recently, RK2-mediated plasmid transfer from E. coli to cyanobacteria has been reported (38). Our results further extend the host range of RK2 conjugation to include anaerobic bacteria. The RK2 conjugation system is an extremely versatile genetic exchange mechanism for the study and manipulation of gram-negative bacteria.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of General Medical Sciences and the National Institute of Allergy and Infectious Diseases. D.G.G. is a Teaching and Research Scholar of the American College of Physicians.

REFERENCES

1. Clark, A., and G. Warren (1979) Ann. Rev. Genet. 13:99-125.
2. Willetts, N., and R. Skurray (1980) Ann. Rev. Genet. 14:41-76.
3. Clewell, D.B. (1981) Microbiol. Rev. 45:409-436.
4. Willetts, N., and B. Wilkins (1984). Microbiol. Rev. 48:24-41.
5. Achtman, M., and R. Skurray (1977) In Microbial Interactions, Receptors and Recognition, Ser. B. V3, J.L. Reissig, ed. Chapman and Hall, London, pp. 233-279.
6. Achtman, M., S. Schwuchow, R. Helmuth, G. Morelli, and P.A. Manning (1978) Molec. Gen. Genet. 164:171-183.
7. Rupp, W., and G. Ihler (1968) Cold Spring Harbor Symp. Quant. Biol. 33:647-650.
8. Ohki, M., and J. Timizawa (1968) Cold Spring Harbor Symp. Quant. Biol. 33:651-657.
9. Clewell, D.B., and D.R. Helinski (1969) Proc. Natl. Acad. Sci., USA 62:1159-1166.
10. Kupersztoch-Portnoy, Y.M., G. Miklos, and D.R. Helinski (1974). J. Bacteriol. 120:545-548.
11. Blair, D.G., and D.R. Helinski (1975) J. Biol. Chem. 250:8785-8789.
12. Lovett, M.A., and D.R. Helinski (1975) J. Biol. Chem. 250:8790-8795.
13. Guiney, D.G., and D.R. Helinski (1975) J. Biol. Chem. 250:8796-8803.
14. Warren, G.J., A.J. Twigg, and D.J. Sherratt (1978) Nature 274:259-261.
15. Dougan, G., and D.J. Sherratt (1977) Mol. Gen. Genet. 151:151-160.
16. Helinski, D.R., and D.B. Clewell (1971) Ann. Rev. Biochem. 40:899-942.
17. Everett, R., and Willetts (1980) J. Mol. Biol. 136:129-150.
18. Collins, J., S. Yanofsky, and D.R. Helinski (1978) Mol. Gen. Genet. 167:21-28.
19. Hershfield, V., H.W. Boyer, L. Chow, and D.R. Helinski (1976) J. Bacteriol. 126:447-453.
20. Guiney, D.G. (1984) J. Infect. Dis. 149:320-329.
21. Bradley, D. (1980) Plasmid 4:155-169.
22. Bradley, D., D. Taylor, and D. Cohen (1980) J. Bacteriol. 143:1466-1470.
23. Guiney, D.G. (1982) J. Mol. Biol. 162:699-703.
24. Willetts, N. (1980) In Molecular Biology, Pathogenicity, and Ecology and Bacterial Plasmids, S. Levy, R. Clowes, and E. Koenig, eds. Plenum Press, New York, pp. 207-216.
25. Guiney, D.G., and J. Ito (1982) J. Bacteriol. 150:298-302.
26. Guiney, D.G., and D.R. Helinski (1979) Mol. Gen. Genet. 176:183-189.
27. Guiney, D.G., and E. Yakobson (1983) Proc. Natl. Acad. Sci., USA 80:3595-3598.
28. Burkardt, J., G. Riess, and A. Puhler (1979) J. Gen. Microbiol. 114:341-348.

29. Ingram, L., M.H. Richmond, and R.B. Sykes (1973) Antimicrob. Ag. Chemother. 3:279-288.
30. Yakobson, E., and D.G. Guiney (1983) Mol. Gen. Genet. 192:436-438.
31. Chikami, G., D.G. Guiney, T. Schmidthauser, and D.R. Helinski (submitted for publication).
32. Villarroel, R., R.W. Hedges, R. Maenhaut, J. Leemans, G. Engler, M. Van Montagu, and J. Schell (1983) Mol. Gen. Genet. 189:390-399.
33. Guiney, D.G., P. Hasegawa, and C.E. Davis (submitted for publication).
34. Barth, P.T., N. Grinter, and D.E. Bradley (1978) J. Bacteriol. 133:43-52.
35. Barth, P.T. (1978) In Plasmids of Medical, Environmental, and Commercial Importance, K. Timmis and A. Puhler, eds. Elsevier/North-Holland, Amsterdam, pp. 399-410.
36. Tinoco, I., O. Uhlenbeck, and H. Levine (1971) Nature 230:362-367.
37. Guiney, D.G., P. Hasegawa, and C.E. Davis. Plasmid (in press).
38. Wolk, C., A. Vonshak, P. Kehoe, J. Elhai (1984) Proc. Natl. Acad. Sci., USA 81:1561-1656.

GENES AND GENE PRODUCTS INVOLVED IN THE SYNTHESIS OF F-PILI

Susan Laine, Deanna Moore, Pushpa Kathir,
and Karin Ippen-Ihler

Department of Medical Microbiology and Immunology
Texas A & M University
College Station, Texas 77840

ABSTRACT

Membrane fractions containing [35-S]methionine labeled proteins synthesized by F*lac* and F*lac tra* mutant strains or by λ*tra* transducing phages expressed in such strains have been analyzed in order to investigate the pathway for synthesis of the F-pilin subunit and the gene products involved in synthesis of F-pili. Our data indicate that the synthesis of a mature F-pilin subunit requires the expression of at least 2 *tra* operon genes in addition to the structural gene for F-pilin, *traA*. In the absence of these activities, *traA* expression results primarily in the synthesis of a polypeptide, Ap14, with an apparent molecular weight of approximately 14,000. We assume this polypeptide corresponds to the direct product of the *traA* gene. In the presence of *traQ* activity, the major detectable product of *traA* is a polypeptide, Ap7(Q), which migrates with an apparent molecular weight of 7,000, suggesting that *traQ* product may process or assist in the processing of Ap14. Polypeptide Ap7(Q) is not, however, mature F-pilin, since it reacts poorly with anti-F-pilus-serum. Synthesis of a polypeptide which appears to be antigenically equivalent to F-pilin and which we assume requires a modification of the F-pilin N-terminus, is detected as synthesis of a polypeptide, Ap7*. This protein migrates slightly more slowly than Ap7(Q) on our polyacrylamide gels. Polypeptide Ap7*, can be efficiently precipitated with F-pilus antiserum, and can be detected in both inner membrane and outer membrane fractions under conditions where assembly of F-pili can occur. These data suggest that Ap7* is the mature F-pilin subunit and is assembled from an inner membrane pool. Synthesis of Ap7* appears to require *traG* activity, but may also be dependent upon additional *tra* activities.

INTRODUCTION

F-pili are filmentous structures elaborated from the surface of cells carrying the conjugative plasmid F. They are required for adsorption of a number of male-specific bacteriophage and can be most easily differentiated from other surface structures by this property. Similar fimbrial appendages are found on the surface of cells which carry a variety of conjugal plasmids, and pili are therefore thought to play an important role in plasmid directed transfer systems. Despite the fact that F-pili appear to be composed of a single subunit (F-pilin), analysis of the activities required to synthesize an F-pilus has proved complex. The genes required to elaborate F-pili are encoded together with other F transfer (tra) genes within a 33 kilobase (kb) region of F DNA. (for review, see Ref. 17 and 18). Expression of at least 14 tra genes is required for F-piliation, and it is likely that additional genes within the cluster remain to be described (3,17). Although the structural gene for F-pilin was determined to be traA, (7), assignment of other functions has been complicated by the difficulty of assaying for intermediates in F-pilin synthesis and assembly. However, the detection of unassembled F-pilin polypeptide in inner membrane preparations of cells harboring F, together with analysis of λtra transducing phage products in Ftra mutant backgrounds permitted us to determine that a gene product (traQ), encoded in a tra operon region 14 kb distal to traA, was required in synthesis of the F-pilin subunit (3,10). In the work described below, additional analyses have been performed to examine the membrane location of unassembled and assembled F-pilin, and to more completely characterize the activity of traQ. We report that an F activity in addition to traQ is required to obtain synthesis of mature F-pilin from traA.

DETECTION OF F-PILIN IN LABELED MEMBRANE PREPARATIONS

Comparison of [35-S] labeled membrane preparations derived from Flac and F- cells and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) permits the detection of only a few F tra operon products. These include the products of traG, traT, and F-pilin which migrate in our gel system at apparent relative molecular weights (M_R) of 100,000, 25,000, and 7,000 respectively (8,9). Since, however, F-pilin subunits are readily detected by this means, we have used such preparations to determine both the membrane location of F-pilin, and the effect of F tra operon mutations on its synthesis and location. In earlier work, we reported that a large number of F-pilin subunits are found in inner membrane preparations, and suggested that F-pili are anchored in the inner membrane and assembled from an inner membrane pool of F-pilin (8). An F-pilin polypeptide could still be detected in membranes of cells carrying Flac tra nonsense or frameshift mutations in any one of the genes traL, traE, traK, traB, traV, traW, traC, traU, traF, or traH, or in the promoter proximal region of traG. Since these mutations prevent

expression of F-pili, this result suggested that these gene products were required for assembly of the pilus structure rather than synthesis of its subunit, although the possibility that one or more of these products effected a change which was not detected on our gels was not excluded (9).

Recent analysis of sucrose density fractionated membranes confirms that such mutations do indeed affect the location of membrane F-pilin. Figure 1 shows a typical density profile of Flac [35-S] methionine labeled membrane proteins after fractionation by SDS-PAGE. As can be seen in this autoradiogram, analysis across the sucrose density gradient revealed significant variations in protein content in samples of different density. Some proteins are remarkably well concentrated in one or 2 fractions, while others are more broadly distributed. Since the preparation and collection of the gradient permits some variation in density of given fractions we distinguish 4 general regions: We label as "OM," fractions of density 1.20 - 1.23 gm/cc which contain the highest concentration of the major host outer membrane proteins. "IM" fractions are density 1.14 - 1.18 gm/cc and contain proteins and enzymatic activities representative of the inner membrane. Intermediate between the OM and IM regions, outer and inner membrane proteins overlap considerably; membranes which appear to represent adhesion zones have been reported at this density (2). Fractions of density 1.14 gm/cc or less contain a different set of proteins, possibly those bound to the cytoplasmic side of the inner membrane. It is interesting to note that we observe these proteins to be lost if membranes are pelleted prior to running the sucrose gradient. They are recovered together with the other membrane proteins after such spins only if the membrane pellet is resuspended by boiling in SDS sample buffer.

Examination of Fig. 1 shows that, as expected, F traT product is concentrated in the OM fractions, while the product of traG is primarily concentrated in the IM fractions (1,9). F-pilin is, however, recovered in significant amounts from fractions spanning the OM, intermediate, and IM regions. In contrast, when membranes of F tra mutants defective in F-pilus assembly are examined (Fig. 2A and 2B) F-pilin is very obviously concentrated in the IM fractions. F-pilin in membranes from an Flac traG24 strain which is defective in F-pilus production exhibits a similar density distribution (Fig. 2C), while much more F-pilin is found in the OM fractions when traG79, an amber mutation which does not prevent F-pilus production is tested (Fig. 2D). Such experiments clearly demonstrate that F-pilin subunits which cannot be assembled accumulate in an inner membrane pool. Since assembled F-pili were not removed from the cell surface of the Flac or Flac traG79 strain prior to preparation of the membrane samples analyzed in these experiments, we assume that the presence of F-pilin in OM samples indicates that subunits which have been completely assembled or which are in the process of assembly are in these fractions. Therefore we can obtain an indicator of F-pilus assembly with such gradients.

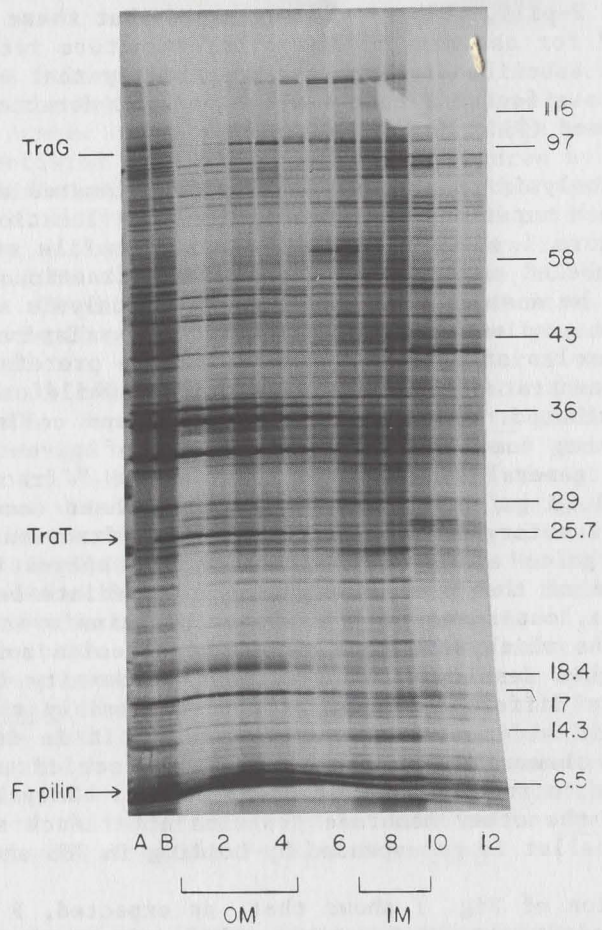


Fig. 1. Autoradiogram of a polyacrylamide gel on which [35-S] methionine labeled membrane proteins from an F*lac* strain were fractionated. Samples analyzed were whole membranes prepared from the female strain, JC3272 (lane A) and its F*lac* derivative, JC3273 (lane B), as well as proteins in membrane pellets derived from each of 12 fractions (labeled in order of decreasing density) of an isopycnic sucrose density gradient of proteins from the F*lac* strain. Fractions corresponding to outer membrane (OM) and inner membrane (IM) densities are indicated. Arrows on the left denote positions of the products of *traG*, *traT*, and F-pilin. Numbers on the right indicate the positions and molecular weights ($\times 10^{-3}$) of marker proteins used, as ascertained from the corresponding stained gel.

Strains, growth media, and procedures were as previously described (8) except that a 10 ml culture of the F*lac* strain was labeled by adding a total of 100 µci [35-S]

Fig. 1. Continued

methionine for a 5 min interval during log phase. The labeled cells were pelleted by centrifugation, resuspended in 1 ml Tris-EDTA buffer [10 mM Tris-HCl, 5 mM EDTA, 1 mM dithiothreitol, pH 7.8], disrupted by passage through a French pressure cell (16,000 psi), and centrifuged for 3 min in an Eppendorf microcentrifuge to remove unlysed cells and large vesicles. A 0.7 ml portion of the supernatant was then placed on top of a sucrose gradient which had been formed by adding successive layers of 0.5 ml 55% and 0.8 ml each of 50, 45, 40, 35, and 30% sucrose in 5 mM EDTA pH 7.5 to an ultracentrifuge tube. After centrifugation for 18 hr at 45,000 rpm in an SW 50.1 rotor, equal fractions of decreasing density were collected from the bottom of the tube. A 50 µl sample was used to determine the refractive index in each aliquot. The remainder was diluted with Tris-EDTA buffer and each fraction was then centrifuged for 18 min at 50,000 rpm in a 70.1 rotor to pellet membrane material. The drained pellets were subsequently resuspended in 50 µl sample buffer and analyzed immediately by SDS polyacrylamide gel electrophoresis using 10-16% exponential gradient gels as described previously except that electrophoresis was at 10 W constant power with cooling (3,8).

EXPRESSION OF *traA*, THE STRUCTURAL GENE FOR F-PILIN

Evidence has accumulated for some time, that the F *traA* gene is the structural gene for F-pilin. The ability of RNA phages to bind to F-pilin is affected by mutation of *traA* (16) or complementation of *traA* mutants with R-100-1 (14). F-pilin purified from a *supF* strain carrying the F*lac* *traA1* (amber) plasmid contained an additional tyrosine (7), and F*lac* *traA* amber strains were unable to synthesize membrane F-pilin (9). However, the direct product of *traA*, a polypeptide which neither comigrated with F-pilin nor appeared to react with F-pilus antiserum was reported to migrate at 13,700 daltons (5,6). This suggested that if *traA* was the structural gene for F-pilin, alteration of its product must be necessary.

The existence and location of an additional *tra* operon activity which participates in F-pilin biosynthesis became evident when it was found that F*lac* *tra* polar mutations or Hfr deletion strains which were defective in expression of the *tra* region between *traF* and *traH* were also deficient in synthesis of membrane F-pilin (8,9). The presence of this function, named *traQ*, could be assayed in UV-irradiated hosts by examining the products of lambda transducing phages carrying the *traA* gene (9). When a female host was used, the major *traA* product was an M_R 14,000 polypeptide which accumulated in the inner membrane. In an F*lac* host, the major *traA* product was an

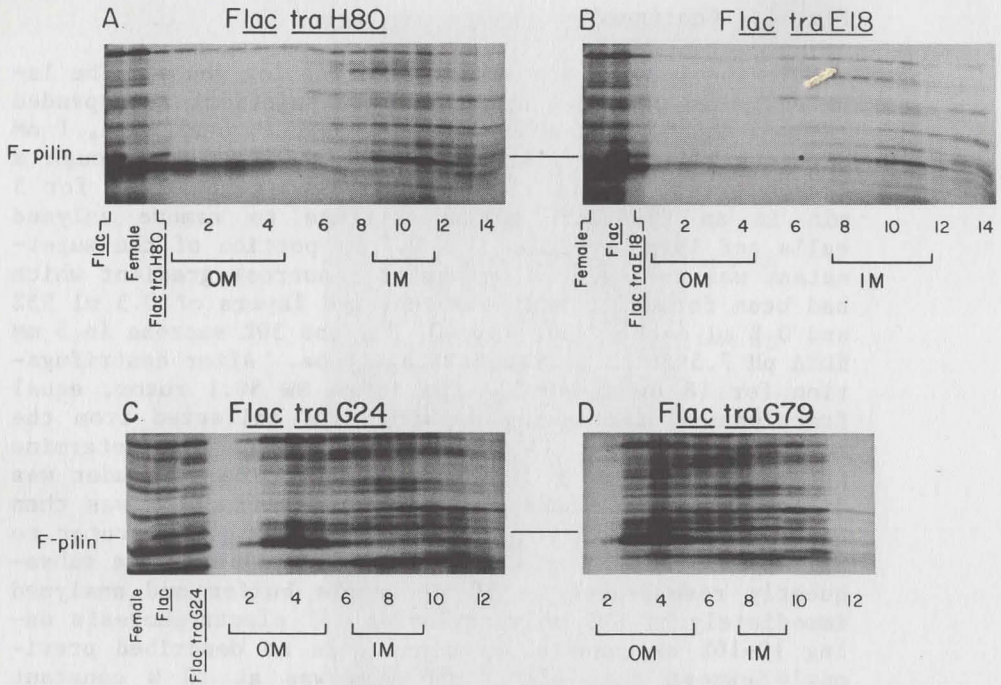


Fig. 2. Lower portions of autoradiograms showing [35-S]methionine labeled proteins present in sucrose gradient fractionated membrane preparations obtained and analyzed as described for Fig. 1. Strains analyzed were derivatives of JC3272 carrying: A. F*lac* *traH80*; B. F*lac* *traE18*; C. F*lac* *traG24*; and D. F*lac* *traG79*. Whole membrane preparations used as controls were from JC3272 (female) or its F*lac* or F*lac* *tra* derivatives as indicated below the autoradiogram. Numbered lanes correspond to samples from sucrose fractions (increase in number indicates decrease in density) from which density fractionated membrane samples were derived. The position at which F-pilin migrates is indicated on the left.

M_R 7,000 protein which comigrated with F-pilin. Similarly, an M_R 7,000 polypeptide was a prominant phage product in experiments in which a phage carrying the entire *traA*-*traH* region was used or when both a TraA$^+$ phage and a phage carrying the *traQ* region were used to coinfect a female host (3).

We have found that examination of density fractionated membrane preparations is also a very useful technique for visualizing *tra* products synthesized after transducing phage infection of UV-irradiated hosts. The background of labeled lambda phage products

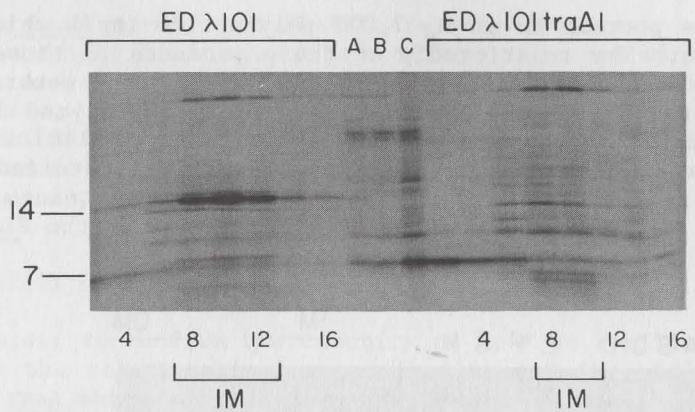


Fig. 3. Lower portion of an autoradiogram of a gel on which membrane proteins labeled during infection of the UV-irradiated female host XK1800 with the λ*traALE* transducing phage EDλ101 (left side) or its *traA1* mutant derivative (right side) were analyzed after fractionation by sucrose density. Preparation, fractionation, and analysis of samples was as described in Fig. 1 except that, in this case, labeling of the transducing phage products in the UV-irradiated host was as described in Ref. 10, and only even-numbered sucrose gradient fractions were analyzed. Also shown are whole cell samples labeled after infection with EDλ101 (lane A), the parental phage, EDλ4 (lane B), or the EDλ101 *traA1* mutant derivative (lane C). Numbers on the left indicate the positions and apparent molecular weight (x 10^{-3}) of the labeled polypeptides present in samples obtained after EDλ101 infection and absent in samples obtained after infection with the EDλ101 *traA1* derivative.

is greatly reduced in these experiments. In Fig. 3 the membrane associated products of EDλ101 [λ p*traALE* (4)] and those synthesized by a *traA1* amber derivative of this phage are compared after infection of the UV-sensitive female host, XK1800. Labeled *traE* product is prominent in the inner membrane preparations from both phage infections, while *traL* product is not obvious, possibly because of the low methionine content of this protein (Frost, Paranchych, and Willetts, in press). It can be seen that the *traA1* mutation prevents the synthesis of a prominent M_R 14,000 EDλ101 encoded inner membrane band. It also prevents the appearance of an M_R 7,000 inner membrane polypeptide. The latter is found in small quantity relative to the M_R 14,000 product, and is only detectable in fractionated membrane samples such as are shown (3). Our interpretation of this result was that, under conditions of over-production and accumulation of the *traA* M_R 14,000 product, (Ap14) the F$^-$ host might inefficiently

process this protein to an M_R 7,000 polypeptide (Ap7) which resembles F-pilin. The relationship of these products to those synthesized in an Flac host is shown in Fig. 4, where inner membrane fractions from the EDλ101 and EDλ101 traA gradients analyzed in Fig. 3 are compared with membrane proteins synthesized by EDλ101 in an Flac traA1 strain. (Background synthesis of F-pilin is avoided in this host since it cannot itself synthesize F-pilin, but can synthesize all other F products prior to the UV treatment.) In the Flac traA1

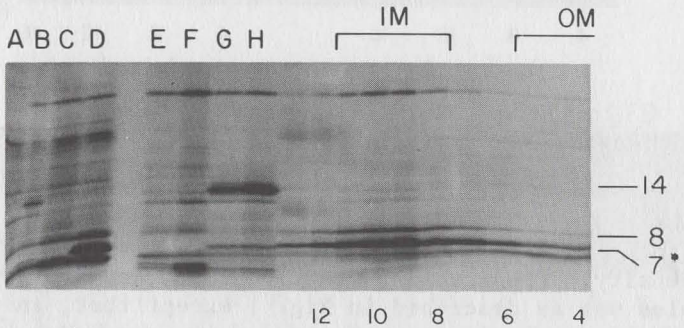


Fig. 4. Proteins labeled during infection with the traALE phage EDλ101 in the UV-irradiated Flac traA1 host, EM 1801. Shown, is the lower portion of the autoradiorgram of a gel on which membrane proteins in fractions 4-13 of a sucrose density gradient were analyzed. In this case, the fractions were placed on the gel such that the density of the fractions increases from left (highest-numbered fractions) to right.

Also analyzed on the same gel, for comparison, are the inner membrane proteins labeled during infection of the female host XK1800 with EDλ101 (lanes G and H) or the EDλ101 traA1 derivative (lanes E and F). These lanes contained fractions 9 and 11 from each of the 2 gradients analyzed in Fig. 3. Labeled proteins detectable in whole cell samples after infection of the UV-irradiated female strain with EDλ101traA1 or EDλ101 are shown in lanes A and B, respectively. Corresponding whole cell samples obtained using the Flac traA1 host are in lanes C and D.

Numbers at the right indicate the position and approximate molecular weight ($\times 10^{-3}$) of the M_R 14,000 traA product readily detected after infection of the female host (lanes B, G, and H), and of the M_R 8,000 and M_R 7000 products detected after infection of the Flac traA1 host. Since this M_R 7000 product can be seen to migrate slightly more slowly than the similar polypeptide detectable in lanes G and H, it is indicated as 7*.

host, the traA products of EDλ101 are very different. Polypeptide Ap14 is no longer detected. A band which comigrates with F-pilin is prominent and can clearly be seen to migrate at a position slightly above the Ap7 polypeptide observed in female hosts. Since it appears to differ from and have a fractionally higher molecular weight than Ap7, we refer to it as Ap7*. In addition, an M_R 8,000 polypeptide (Ap8) appears.

PRECIPITATION OF TraA PRODUCTS WITH ANTISERUM

In order to confirm the identity of Ap7* as F-pilin and to investigate the relationship between F-pilin and the additional products of traA which could be detected in our system we raised rabbit antiserum to purified F-pilin and examined the membrane products of EDλ101 which it precipitated. When the traA gene of EDλ101 was expressed in an Flac traA1 host, the serum could be used to efficiently immunoprecipitate both Ap7* (F-pilin) and Ap8. However, we were unable to detect either Ap14 or Ap7 in anti-F-pilin precipitates of EDλ101 expressed in female cells (3). Since the traA1 amber mutation affected the synthesis of all of these polypeptides, and suppression of traA1 results in F-pilin with an altered amino acid content (7), these proteins must contain a common amino acid sequence. Our result could be explained then, only if this amino acid sequence is insufficient to comprise the major antigen of F-pilin. Evidence that, in fact, the major antigen of F-pilin might derive from a modification of its amino acid sequence had been provided by the work of Worobec et al. (19) who demonstrated that the major antigen of EDP208 pilin is the blocked N-terminus of this polypeptide. We surmised that F-pilin might have a similarly blocked N-terminus.

Nevertheless, it seemed reasonable to predict that a common amino acid sequence shared by all traA products should be detectable with anti-F-pilus serum if our system could be made sufficiently sensitive. Therefore, we constructed strains which would make these products in greater quantity by cloning a PstI fragment containing traALE from pBE268 into the PstI site of pLA2311 (12,13). In the plasmid (pKI301) thus constructed, genes traALE are located downstream from the lambda promoter P_L. A strain expressing λCI857 repressor and carrying pKI301 grows normally at 30°C. When it is shifted to 42°C, an immediate increase in pKI301 products and concommitant decrease in host protein synthesis is observable. Within 30 min, synthesis of the products of pKI301 including the products of traA and traE, predominates completely over host protein synthesis; during this time, however, total protein synthesis diminishes. Cellular viability is eventually lost (D. Moore, unpub. data). Figure 5 shows [35-S]methionine membrane proteins obtained when an Flac traA1 (lane 6) or a female (lane 7) host carrying pKI301 were labeled for 5 min in the interval 10-15 min after induction at 42°C. Whole-cell samples of EDλ101 (traALE) products are shown in lanes 1

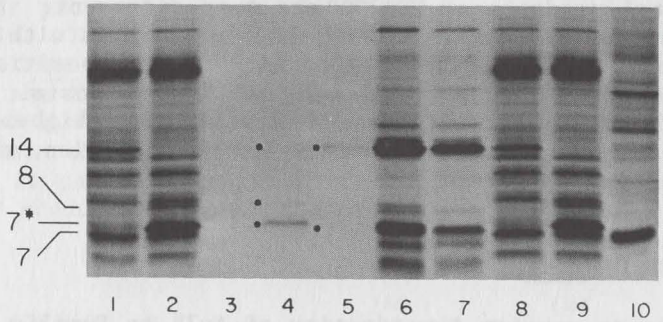


Fig. 5. Analysis of antibody precipitable proteins synthesized by pKI301 in a F⁻ and in an Flac traAl host. pKI301 is a derivative of the vector pLa2311 (12) into which a PstI fragment carrying genes traALE has been inserted. Expression of the tra segment occurs from lambda P_L, at 42°C. Analyses of whole membrane samples of [35-S]methionine labeled proteins synthesized in the interval 10-15 min after induction at 42°C are shown for a pKI301/F⁻ strain (lane 7), a pKI301/Flac traAl strain (lane 6) and for the pLa2311/Flac traAl control (lane 10). Analyses of the proteins precipitated from these samples with anti-F-pilus serum and IgSorb are also shown: pKI301/F⁻, lane 5; pKI301/Flac traAl, lane 4; pLa2311/Flac traAl, lane 3. Whole cell samples of labeled EDλ101 products obtained after infection of a female host (lanes 1 and 8) or an Flac traAl host (lanes 2 and 9) were also analyzed for reference. Numbers on the left indicate the apparent molecular weight of the corresponding antibody precipitable bands (marked by dots) in lanes 4 and 5.

Membrane preparation and antibody precipitation was performed as described previously, except that only 1 ml samples of the plasmid-containing strains were used, and cells were disrupted by sonication (3).

and 8 (F⁻ host) and lanes 2 and 9 (Flac traAl host) for comparison. It is clear that Ap14 is synthesized from pKI301 in abundance in both host backgrounds. The Ap7 polypeptide is also made in the female host, while Ap7* together with Ap8 are synthesized in the Flac traAl strain. It is further demonstrated (lanes 4 and 5) that anti-F-pilus serum can be used to immunoprecipitate all of these proteins, confirming a relationship in their structure which we assume derives from the common amino acid sequence we predicted they must have. As we had observed in the λtra system, Ap7* was clearly the most efficiently precipitated polypeptide in keeping with our identification of this polypeptide as the antigenically mature F-pilin subunit.

PRODUCTS OF EDλ134 [tra(Y)ALEKBPVWCUNFQH(G)]

As indicated above, we have reported previously that no M_R 7,000 band resembling F-pilin could be detected in membranes of Hfr mutants defective in expression of the region between traF and traH. We postulated that an activity stemming from a tra gene (traQ) in the traF-traH region was required for F-pilin synthesis (10). When the products of a TraA⁺ phage coinfected with a TraQ⁺ phage, or the products of a large transducing phage (EDλ134) which expresses the entire traA - traH region were examined, an M_R 7,000 polypeptide became a major product of traA (3). These results are illustrated by experiments such as that shown in Fig. 6 in which inner membrane associated polypeptides synthesized by EDλ134 during infection of F⁻ cells were analyzed. The lambda products synthesized by the parental phage, EDλ4, which were analyzed simultaneously, are also shown. Comparison of the products of EDλ4 and EDλ134 permits the detection of many tra operon proteins. Some of these have been provisionally identified (Moore and Ippen-Ihler, unpub. data). Further analysis of the products of cloned subsegments of DNA from the tra operon as well as the products of additional transducing phages is currently in progress to determine the exact tra regions specifying others. Examination of the region in which F-pilin migrates shows clearly, that an M_R 7,000 band is synthesized by EDλ134 and that lesser quantities of an M_R 8,000 protein are also readily detectable. These same polypeptide bands appeared to be synthesized whether an Flac traAl or an F⁻ host was used, and to comigrate with the products of the TraA⁺ phage EDλ101 which are synthesized in an Flac traAl host. Thus, such results initially suggested that the tra genes expressed from the TraA⁺TraQ⁺ phage EDλ134 could accomplish synthesis of F-pilin (3). However, a close examination of well-resolved gels on which EDλ134 products made in male and female hosts were compared, as well as analysis of anti-F-pilus serum precipitated proteins has shown that they are not identical.

As can be seen in Fig. 7, the anti-F-pilus serum worked efficiently in immunoprecipitation of AP7* (F-pilin) together with Ap8 when the male host was used for the EDλ134 infection. In contrast, the M_R 7,000 and 8,000 polypeptides made in F⁻ cells were very poorly precipitated. Again, when good resolution is obtained, the M_R 7,000 EDλ134 product can be seen to migrate slightly faster than F-pilin, resembling Ap7 rather than Ap7*. Since we do not known if it is exactly identical to the Ap7 polypeptide observed in A⁺Q⁻ infections we refer to it as Ap7(Q). It is obvious that the tra gene products expressed from EDλ134 cannot, alone, accomplish addition of the major antigen of F-pilin. That is, EDλ134 products appear to accomplish efficient synthesis of Ap7(Q) and AP8, but not Ap7*.

This is intriguing because EDλ134 carries tra(Y)ALEKBPVWCUNFQH(G), and, therefore, expresses almost all of the genes required for F-pilus assembly. It thus seemed possible that addition of the

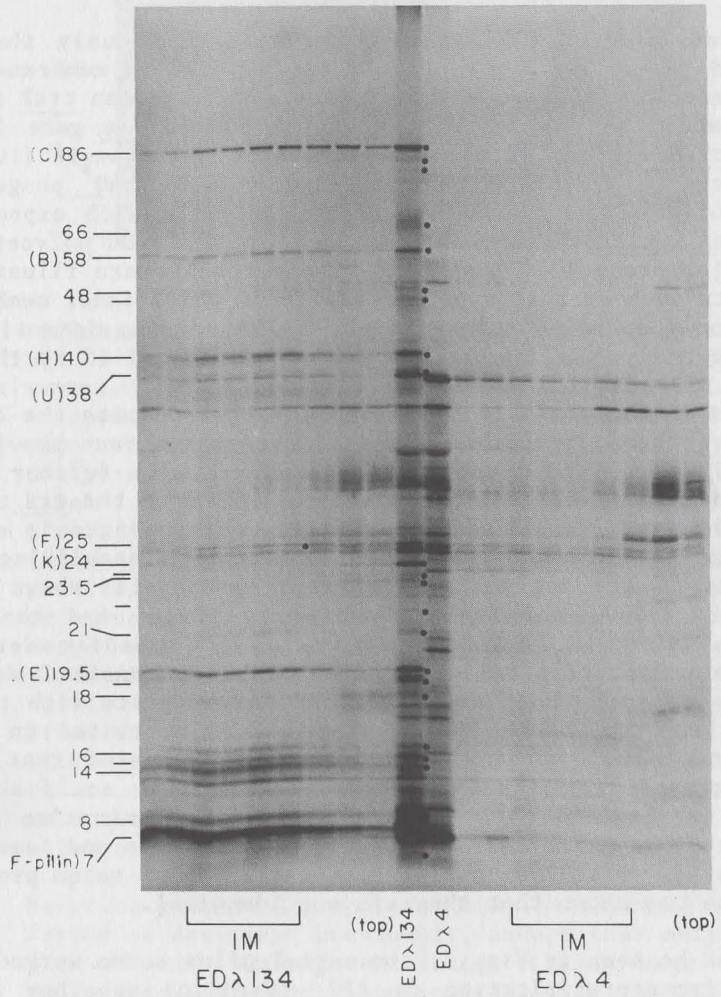


Fig. 6. Analysis of [35-S]methionine labeled proteins synthesized after infection of UV-irradiated XK1800 with either EDλ134, which carries a tra(Y)ALEKBPVWCUNFH(G) segment or its parental phage, EDλ4 (3,4). Whole membrane samples are shown in the center lanes; membrane samples obtained after sucrose density fractionation are shown on the left for EDλ134, on the right, for EDλ4. Only fractions of intermediate to light density were analyzed on this gradient. Black dots indicate bands detected in membranes from EDλ134 infected cells which were not present after EDλ4 infection. Numbers on the left indicate the apparent molecular weight (x 10^{-3}) of these bands; the tra cistrons thought to encode some of these products are also indicated.

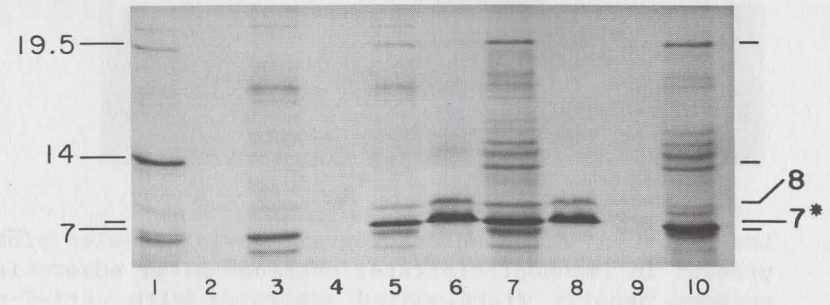


Fig. 7. Lower portion of an autoradiogram showing analysis of membrane proteins labeled during transducing phage infection and the proteins immunoprecipitated from these preparations after adsorption to anti-F-pilus serum. Samples analyzed were: membranes (lane 1) and immunoprecipitate (lane 2) from EDλ101 infected F^- cells; membranes (lanes 3,5, and 7) and immunoprecipitates (lanes, 4,6 and 8) from Flac traA1 cells infected with EDλ4 (lanes 3 and 4), EDλ101 (lanes 5 and 6) and EDλ134 (lanes 7 and 8); and the immunoprecipitate (lane 9) and membrane preparation (lane 10) from EDλ134 infected F^- cells. Experimental procedures were as described previously (3). Numbers at the side of the figure indicate the apparent molecular weight (x 10^{-3}) and positions of the product of traE (19.5) and polypeptides associated with traA expression.

F-pilin major antigenic group might be associated with the assembly of F-pilin into F-pili, a process which could occur in the Flac traA1 host, but not in the F^- host. Examination of EDλ134 membrane products on sucrose gradients indicated that while most of the F-pilin polypeptide synthesized in either the F^- or Flac traA1 host could be found in the inner membrane, a significant amount could also be detected at densities associated with intermediate and outer membrane when the Flac traA1 host was used, that is, in samples we consider to have contained assembled F-pilin (P. Kathir, unpub. data). Therefore, we tested the capacity of our anti-F-pilus serum to precipitate F-pilin from membrane preparations of different density. The results are shown in Fig. 8. When the Flac traA1 host was used, the antiserum precipitated F-pilin from all portions of the gradient. That is Ap7* was found not only in membrane samples of outer membrane density, but also, indeed most heavily, was concentrated in samples of inner membrane density. Therefore, while it is still possible that addition of the antigenic group occurs as a condition of assembly, it appears to occur in the inner membrane, and the unassembled inner membrane pool of F-pilin in male cells carries this antigenic modification.

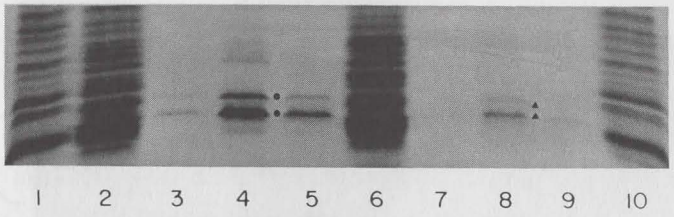


Fig. 8. Lower portion of an autoradiogram showing labeled proteins present in immunoprecipitates obtained after adsorption of sucrose density fractionated membranes with anti-F-pilus serum. Shown are the immunoprecipitates from (outer) membranes of density 1.21-1.23 gm/cc (lanes 3 and 7), (inner) membranes of density 1.14-1.18 gm/cc (lanes 4 and 8), and light fractions of density 1.07-1.11 gm/cc (lanes 5 and 9). Samples were labeled during EDλ134 infection of either the Flac traAl host (lanes 3-5) or the F⁻ host (lanes 7-9). Dots indicate the polypeptides Ap8 and Ap7* synthesized in the Flac traAl host; ▲, the polypeptides Ap8 and Ap7(Q) synthesized in the female host. Also shown are whole cell preparations of proteins labeled after EDλ4 or EDλ134 infection of the Flac traAl host (lanes 1 and 2, respectively) or of the female host (lanes 10 and 6, respectively).

DISCUSSION

Our data allow us to conclude that biosynthesis of F-pilin requires expression of at least 2 tra activities in addition to expression of the structural gene, traA. An M_R 14,000 product (Ap14) is the major product of traA when other tra activities are not present. A small amount of an M_R 7,000 polypeptide which we presume stems from inefficient host processing of Ap14 is also observable. Ap14 is efficiently replaced by an M_R 7,000 polypeptide [Ap7(Q)] in $TraA^+$ $TraQ^+$ systems, while yet another F activity must also be present in order to synthesize the modified polypeptide (Ap7*) which is antigenically identical to F⁻pilin. An M_R 8,000 polypeptide (Ap8) is also observed in $TraA^+$ $TraQ^+$ systems and may be an intermediate or byproduct of the traQ reaction. These results are summarized in Fig. 9.

The Nature of the traQ Reaction

We have previously suggested that traQ product functions in a processing reaction in which the direct product of traA (Ap14) is cleaved (3,10). Although the exact nature of the traQ-mediated reaction is still unclear, this model remains the most straightforward explanation of our data. The DNA sequence of traA, recently determined by Frost, Parenchych, and Willetts (J. Bact., in press), can be interpreted quite consistently with our hypothesis. We assume

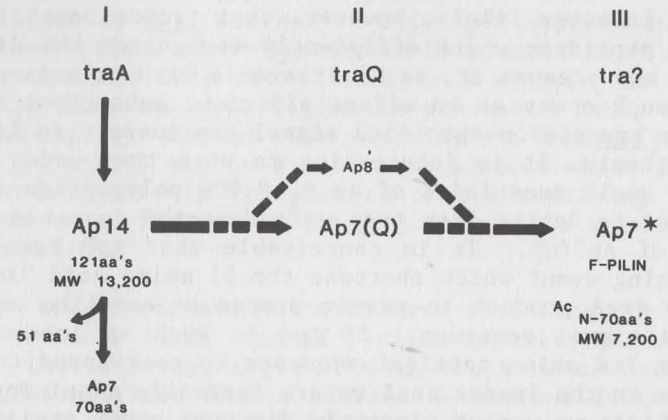


Fig. 9. Diagram showing the possible pathway of F-pilin synthesis. Expression of traA in a female host leads to synthesis of Ap14 from which small amounts of Ap7 can be obtained. When traQ activity is present Ap14 appears to be converted to Ap7(Q) and small amounts of Ap8 are also detected. If additional tra activities are also present, a polypeptide, Ap7*, which appears to be antigenically equivalent to F-pilin is synthesized. Numbers of amino acids per polypeptide, and the acetylated N terminus for F-pilin are surmised for these products based on the data of Frost, Paranchych, and Willetts (J. Bact., in press). Dotted lines indicate that the relationship of Ap8 and Ap7(Q) to the pathway is not yet clear (see text).

polypeptide Ap14 corresponds to the 121 amino acid polypeptide these authors predict to be encoded by the traA reading frame. Polypeptides Ap7 and Ap7(Q) correspond well in size to the 70 amino acid C terminal portion of the traA gene coded product they have ascribed to F-pilin. Since both a core sequence of hydrophobic amino acids and a typical signal peptidase recognition site (11) appear in the sequence immediately preceding those amino acids which match the F-pilin N-terminus, Frost, Paranchych, and Willetts have suggested that a signal peptidase cleavage can occur at the F-pilin N terminus, i.e., between amino acids 51 and 52 of the traA product. We assume that our ability to detect Ap7 reflects this occurrence. Since, however, we observed very little Ap7 polypeptide to be made when traA was expressed in an F⁻ host, and Ap14 always predominated under such conditions, it would appear that host enzymes can accomplish this processing only very inefficiently. Indeed, it seems likely that the appearance of Ap7 is a consequence of overproduction of Ap14 in the absence of the tra products with which it would normally associate.

It is possible to imagine a number of different mechanisms through which traQ product might accomplish the efficient production

of Ap7(Q). It seems likely, however, that traQ product either: 1) is a signal peptidase which efficiently recognizes the direct product of traA and cleaves it, or 2) interacts with or acts on the traA product in such a way as to effect efficient subsequent recognition by a host or tra operon-specified signal peptidase. In light of the latter hypothesis, it is interesting to note that under TraA$^+$ TraQ$^+$ conditions, small quantities of an M_R 8,000 polypeptide (Ap8) which also appeared to derive from traA were detected in concert with the appearance of Ap7(Q). It is conceivable that Ap8 results from a traQ processing event which shortens the 51 amino acid "leader" portion of the traA product to permit proper orientation and recognition of its signal sequence. If Ap8 is such an intermediate, we would expect its amino terminal sequence to correspond to an internal sequence in the leader, and we are currently examining this possibility. Since we cannot presently discount other explanations for Ap8, such as aberrent migration or host modification of Ap7(Q), and it is possible that either one or both of these proteins are intermediates in synthesis of Ap7*, the dotted lines in Fig. 9 reflect this ambiguity. Our present assumption is that Ap7(Q), like Ap7, results from a cleavage between amino acids 51 and 52. Since traQ mediated processing could differ from host processing, the N-terminal sequence of Ap7(Q) is also being analyzed.

It is important to note that, at this point, we have not been able to demonstrate precursor relationships among the traA products. Ap14, for example, is typically detectable only when synthesized in excess to traQ expression, and under these circumstances appears stable in chase experiments. Similarly, we do not yet know if Ap8 or Ap7(Q) are actual precursors to Ap7*. It is possible that one or both are end products made only when appropriate interactions with other tra proteins are precluded. Attempts to reversibly inhibit the synthesis of F-pilin using ethanol or a secA(ts) host were unsuccessful (Laine, unpub. data). It seems likely that translation of traA and tra mediated processing of its product are tightly coupled reactions. We are approaching this problem by constructing sets of compatible plasmids on which expression of various tra genes can be induced.

The traQ product itself has not yet been identified. Our most recent experiments indicate that traQ activity can be expressed from a 6.45 kb SmaI fragment which includes F DNA between coordinates 77.45 and 83.9 on the map of Skurray and Willetts (15,17). Although the activities of traU, traN and traF which are carried in this segment do not appear to be involved in processing the traA product, our clone expresses at least 2 additional tra polypeptides (Moore, Wu, and Ippen-Ihler, unpub. data). Further experiments are therefore necessary to determine whether the traQ-mediated reaction requires one or both of these polypeptides.

The nature of the modification reaction. It is apparent from our data that when all tra genes are expressed, the final product of the

traA gene is not Ap7(Q), but Ap7*. Only Ap7* appears antigenically equivalent to F-pilin. Ap7* appears to be a modified form of Ap7(Q) which is synthesized only if yet another F activity in addition to that stemming from the traQ region is present. Thus, Ap7* may be derived from Ap7(Q) through the activity of a modifying enzyme. Alternatively, Ap7(Q) may be an aberrent product which is made when the traQ product acts in the absence of the modifying enzyme. In any case, our assay for the latter activity is the synthesis of Ap7* rather than Ap7(Q).

Frost, Paranchych, and Willetts have also recently established that the N-terminal amino acid of F-pilin is acetylated, (J. Bact., in press). This finding, together with the work of Worobec et al. (19) which demonstrated that a similar N terminus is the major antigen of EDP208 pilin, strongly suggests that the antigenic difference we detect in comparison of Ap7(Q) and Ap7* reflects the acetylation of the F-pilin N terminus. As we are able to find Ap7* in the inner membrane, our data further suggest that the acetylation occurs during the synthesis of the F-pilin inner membrane pool, prior to or in concert with assembly of F-pili. Although the system in which we detected the addition of antigen also permitted assembly of F-pili, it seems likely that a complete assembly apparatus is not an absolute requirement for completion of the modification. We have previously shown that the M_R 7,000 product of a TraA$^+$ phage expressed in Flac hosts carrying mutations in traB, traV, traW, traC, traU, or traF migrates more slowly than the polypeptide we now call Ap7. We presume, therefore, that synthesis of Ap7* occurs despite such mutations. Nevertheless, we have not yet completed antibody assays for Ap7* in such mutant strains, and our earlier experiments in which we reported the presence of F-pilin polypeptide in the membranes of other Flac tra mutant hosts would not necessarily have distinguished Ap7* from Ap7(Q). Additional experiments are, therefore, needed to determine the minimal number of tra gene products necessary to accomplish the modification reaction. However, the gene products expressed by EDλ134, which carries a very large segment of the tra operon, were clearly insufficient. It is obvious that some Flac product, not expressed by EDλ134 is required. Yet, mutation or deletion of most of the tra genes which are not expressed from EDλ134 does not affect F-piliation. If we assume that only Ap7* can be assembled into F-pili, the gene products of traM, traS, traT, traD, traI, or traZ cannot be involved. The most obvious remaining candidates are traG and traJ. Our preliminary experiments suggest that it is the N-terminal portion of traG product which has been shown to be required for piliation that is also necessary for synthesis of Ap7*. The modification appears to progress in an Flac traJ90 background (unpub. results). However, synthesis of competing unlabeled F-pilin has complicated interpretation of antiserum precipitates of EDλ134 products synthesized in Flac traG, and other Flac hosts. Therefore, although traG product appears to be required in the synthesis of Ap7*, further experiments are required to establish its exact role. If, indeed, one function of traG product is to

acetylate the F-pilin N terminus, it is interesting to speculate that the C-terminal portion of the traG polypeptide, [which is not required for expression of F-pilin, but is essential for mating pair stabilization and DNA transfer (See Ref. 17 for summary of evidence)] might modulate a mating-stimulated alteration of this reaction.

F-pilus assembly. In addition to the products of traA, traQ, and traG, at least 10 additional tra genes are known to be required in order to accomplish expression of an F-pilus on the cell surface. Since these genes span a region of the tra operon which has not yet been genetically saturated and appears to encode more gene products than could be encoded by known genes (3), it seems probable that the number of gene products involved in F-pilus assembly, in fact, exceeds this number. As a working model, we propose that these proteins interact in a trans-membrane complex which creates an F-specific adhesion between the inner and outer membrane of the host. We imagine that this adhesion complex functions in assembly and disassembly of F-pilin from the inner membrane and in conduction of nucleic acid during conjugation and infection by F-pilus specific bacteriophages. Although we find it experimentally convenient to determine the associations of these proteins with host inner and outer membrane material, it is important to comment that we imagine that these associations reflect orientations of tra proteins which are associated with each other in a complex which spans both. Although, as yet, we know very little about the individual functions of the numerous tra products associated with F-piliation, we expect that the techniques we have developed for analysis of the pathway of F-pilin synthesis will prove useful in identifying these products and analyzing the interactions which occur between these proteins.

ACKNOWLEDGMENT

We are grateful to Laura Frost, William Paranchych and Neil Willetts for sharing their unpublished results, and providing us with the DNA sequence of traA prior to its publication. We thank David McMurray for his assistance in the preparation of anti-F-pilus serum. Our work was supported by Public Health Service Grant AI 14426 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Achtman, M., N. Kennedy, and R. Skurray (1977) Cell-cell interactions in conjugating E. coli: Role of traT protein in surface exclusion. Proc. Natl. Acad. Sci., USA 74:5104-5108.
2. Bayer, M., G. Costello, and M. Bayer (1982) Isolation and partial characterization of membrane vesicles carrying markers of the membrane adhesion sites. J. Bact. 149:758-767.

3. Ippen-Ihler, K., D. Moore, S. Laine, D.A. Johnson, and N.S. Willetts (1984) Synthesis of F-pilin polypeptide in the absence of F traJ product. Plasmid 11:116-129.
4. Johnson, D.A., and N.S. Willetts (1983) λ-Tranducing phages carrying transfer genes isolated from an abnormal prophage insertion into the traY gene of F. Plasmid 9:71-85.
5. Kennedy, N., L. Beutin, M. Achtman, R. Skurray, W. Rahmsdorf, and P. Herrlich (1977) Conjugation proteins encloded by the F sex factor. Nature 270:580-585.
6. Manning, P.A., and M. Achtman (1974). Cell to cell interactions in conjugating E. coli: The involvement of the cell envelope. In Bacterial Outer Membranes, M. Inouye, ed. Wiley, New York, pp. 409-447.
7. Minkley, Jr., E.G., S. Polen, C.C. Brinton, Jr., and K. Ippen-Ihler (1976) Identification of the structural gene of F-pilin. J. Mol. Biol. 108:111-121.
8. Moore, D., B.A. Sowa, and K. Ippen-Ihler (1981) Location of an F-pilin pool in the inner membrane,. J. Bact. 146:251-252.
9. Moore, D., B.A. Sowa, and K. Ippen-Ihler (1981) The effect of tra mutations on the synthesis of the F-pilin membrane polypeptide. Mol. Gen. Genet. 184:260-264.
10. Moore, D., B.A. Sowa, and K. Ippen-Ihler (1982) A new activity in the F tra operon which is required for F-pilin synthesis. Mol. Gen. Genet. 188:459-464.
11. Perlman, D., and H.O. Halvorson (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. 167:391-409.
12. Remaut, E., P. Stanssens, and W. Fiers (1981) Plasmid vectors for high efficiency expression controlled by the P_L promoter of coliphage lambda. Gene 15:81-83.
13. Thompson, R., and M. Achtman (1978) The control region of the F Sex Factor DNA transfer cistrons: Restriction mapping and DNA cloning. Mol. Gen. Genet. 165:295-304.
14. Willetts, N.S. (1971) Plasmid specificity of two proteins required for conjugation in E. coli K-12. Nature New Biol. 230:183-85.
15. Willetts, N., and G. Johnson (1979) R. SmaI cleavage map of the transfer operon of the E. Coli K12 sex factor F. Genet. Res. Camb. 34:195-202.
16. Willetts, N.S., P.H. Moore, and W. Paranchych (1980) Variant pili produced by mutants of the Flac plasmid. J. Gen. Microbiol. 117:445-464.
17. Willetts, N., and R.A. Skurray (1980) The conjugation system of F-like plasmids. Ann. Rev. Genet. 14:41-76.
18. Willetts, N., and B. Wilkins (1984) Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. 48:24-41.
19. Worobec, E.A., A.K. Tanja, R.S. Hodges, and W. Paranchych (1983). Localization of the major antigenic determinant of EDP208 pili at the N-terminus of the pilus protein. J. Bact. 153:955-961.

GENETICS OF CLINDAMYCIN RESISTANCE IN *BACTEROIDES*

C. Jeffrey Smith,[1] Melissa C. Liechty,[2]
Jeanette L. Rasmussen,[2] and Francis L. Macrina[2]

[1]Laboratory of Molecular Microbiology
National Institute of Allergy and Infectious Diseases
Fort Detrick
Frederick, Maryland 21701

INTRODUCTION

The colonic *Bacteroides* species are numerically predominant members of the human intestinal tract microflora (2,6). In addition to their presence as indigenous flora, these organisms are significant opportunistic pathogens implicated in a variety of infectious processes (1). Clindamycin, a lincosamide antibiotic, is used extensively as a first line drug for the treatment of anaerobic infections caused by *Bacteroides* species. However, the increasing emergence of lincosamide-macrolide (LM) (e.g., clindamycin-erythromycin) resistant *Bacteroides* in the clinical setting (18) has severely compromised routine antimicrobial chemotherapy.

Following the appearance of clindamycin-resistant *Bacteroides* strains, there were several reports demonstrating conjugal transfer of antibiotic-resistance among *Bacteroides* species, and the first *Bacteroides* R-plasmids were described (12,14,20,21,23,24). Thus far, 3 *Bacteroides* R-plasmids have been described, and a summary of their relevant properties is presented in Tab. 1. The 3 plasmids vary widely in size and represent distinct, self-transmissible replicons; however, they each encode high-level resistance (> 100 µg per ml) to the antibiotics clindamycin, erythromycin, and lincomycin. Based on DNA-DNA hybridization studies, a related LM-

---

[2] Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298.

Tab. 1. Summary of properties of Bacteroides R-plasmids.

Host Strain	Plasmid	Molecular Size (kb)	Phenotype[a]	Geographic Origin	Ref.
B. fragilis TMP10	pBFTM10	14.6	LM resistance conjugal transfer	Los Angeles, CA	(21)
B. fragilis V479-1	pBF4	41	LM resistance conjugal transfer	Paris, France	(23)
B. ovatus IB106	pBI136	82	LM resistance conjugal transfer	Paris, France	(15)

[a]The phenotype of LM resistance indicates resistance to more than 100 µg per ml of clindamycin or erythromycin.

resistance determinant has been found associated with all 3 plasmids (14,15). Although apparently widespread, there is a lack of information regarding the mode of dissemination of this antibiotic-resistance determinant.

In addition to the R-plasmids found in Bacteroides, several laboratories have described strains that conjugally transfer an LM-resistance phenotype by a mechanism which does not appear to involve detectable plasmid DNA (8,10,19). The cellular location of these "nonplasmid associated" resistance determinants is not yet known but they may be chromosomally located. Hybridization studies with these strains indicate that the LM-resistance determinant is similar to the one associated with the Bacteroides R-plasmids described in Tab. 1 (5,9,10).

By way of example, using the recently described R-plasmid pBI136 (15), this paper will summarize much of the current information regarding transmissible LM-resistance in the Bacteroides. Data will be presented on the molecular structure of both the plasmid-mediated and nonplasmid associated LM-resistance determinants.

RESULTS AND DISCUSSION

The Antibiotic Resistance Transfer Event

Bacteroides ovatus IB106 is a clinical isolate resistant to more than 100 µg per ml of clindamycin and erythromycin. Transfer of this LM-resistance phenotype to 2 suitably marked recipient strains was demonstrated by cocultivation of cells on nitrocellulose filters placed on nonselective agar plates in an anaerobic chamber. As shown in Tab. 2, the transfer frequency ranged from 10^{-7} to 10^{-5} transconjugants per input donor cell. The transconjugant clones obtained from these matings were also transfer proficient at comparable frequencies (Tab. 2, expt. 3 and 4). The transfer frequencies observed were in the range reported for other Bacteroides systems (10,21,23). The frequency of transfer was somewhat dependent upon the Bacteroides species participating in the matings (Tab. 2; compare expt. 1 and 2, and expt. 3 and 4). Although the basis for this difference is not understood, other reports also have shown differences in transfer frequencies of interspecies matings (23).

Available evidence suggests that the transfer of LM resistance from IB106 occurs by a mechanism that fits the criteria for a conjugation-like event. Using previously published methods (16,23), the transfer process was found to be insensitive to the action of DNase I and it required cell-to-cell contact between donor and recipient strains. Moreover, neither chloroform-treated donor cells nor donor cell-free filtrates could mediate the antibiotic resistance transfer event. In addition, transfer did not occur in liquid broth cultures of donor and recipient strains. These results are characteristics of all Bacteroides transfer systems described to date (5,16,19,21,23). There are no known reports of genetic exchange mediated by transformation or transduction in Bacteroides.

Plasmid Analysis of IB106

B. ovatus IB106 contains 2 plasmid species: pBI106, 44 kilobases (kb) and pBI136, 82 kb (Fig. 1). Detection of pBI136 was initially complicated by a relatively high concentration of pBI106 in the plasmid preparations. Estimates based on the fluorescence of

Tab. 2. Conjugal transfer of LM resistance among Bacteroides strains.

Expt.[a]	Donor Strain	Recipient Strain	Selective[b] medium	Transfer[c] frequency
1.	B. ovatus IB106	B. fragilis 638	Cc Rf	5×10^{-7}
2.	B. ovatus IB106	B. uniformis V528	Cc Rf	1×10^{-5}
3.	B. fragilis IB131	B. ovatus V211	Cc Rha	2×10^{-7}
4.	B. uniformis IB128	B. ovatus V211	Cc Rha	4×10^{-5}

[a]The transconjugants retained for further study from each experiment were as follows: Expt. 1, B. fragilis IB126 and IB131; Expt. 2, B. uniformis IB128 and IB129; Expt.3, B. ovatus IB136; and Expt. 4 B. ovatus IB137.

[b]The selective medium for experiments 1 and 2 was supplemented Brain Heart Infusion agar (16) containing 5 µg per ml clindamycin (Cc) and 25 µg per ml rifampicin (Rf). For experiments 3 and 4 a defined medium (16) containing 10 µg per ml clindamycin, and rhamnose (0.2%; Rha) as sole energy source was used for selection.

[c]Transfer frequency was estimated by averaging results from at least three independently performed experiments. Transfer frequency is the number of LM resistant transconjugants per viable input donor cell.



Fig. 1. Plasmid DNA isolated from IB106 and LM resistant transconjugants. IB106 (lane 1), IB128 (lane 2), IB131 (lane 3), IB137 (lane 4), IB136 (lane 5), and V517 (lane 6) plasmid size reference molecules (7). For derivation of transconjugant strains see the footnotes to Tab. 2. Plasmid DNA was purified by CsCl-ethidium bromide ultra-centrifugation of cleared lysates (4). (Ori), origin of the gel; and (chr), region corresponding to chromosomal DNA.

diluted plasmid samples electrophoresed on agarose gels and stained with ethidium bromide indicated that pBI106 was present in at least 20-fold higher concentration than pBI136 (data not shown).

Examination of LM-resistant transconjugants for plasmid DNA generally resulted in a plasmid species similar in size to pBI136 (Fig. 1). In most strain backgrounds (e.g., B. uniformis IB128 and B. fragilis IB131) the presence of pBI136 was difficult to detect and sometimes impossible to visualize by ethidium bromide staining. Samples from the equivalent of 100 ml or more of culture were usually required for analysis on agarose gels. Plasmid pBI136 DNA more routinely was obtained in higher amounts when it was present in the B. ovatus V211 strain (e.g., strains IB136 or IB137). The yields of plasmid DNA were still significantly lower than those seen for pBF4 and pBFTM10 in the same genetic background.

Due to this, inconsistency of plasmid isolation from transconjugant strains, a role for pBI136 in LM-resistance transfer, was demonstrated by filter blot hybridization studies. EcoRI restricted total cellular DNA from IB106 and transconjugants was probed with either pBI106 or pBI136 plasmid DNA and the resulting autoradiographs are shown in Fig. 2. These results clearly show that pBI106 was not involved in either LM resistance or its transfer since the original donor IB106 was the only strain that possessed sequences homologous to pBI106 DNA (Fig. 2A). When pBI136 was used as the probe, strain IB106 and all LM-resistant transconjugants exhibited homologous sequences which comigrated with purified, EcoRI-digested pBI136 (Fig. 2B). No such hybridizing sequences were observed in the original recipient strains. These data suggested a stable inheritance of pBI136 among all transconjugants including those in which the plasmid was not readily detected.

Besides the 7 EcoRI fragments of pBI136, each of the transconjugants contained an additional fragment which hybridized weakly to the pBI136 probe and migrated between the EcoRI F and G fragments. The origin of these additional sequences is not known but their migration patterns varied consistently depending on the strain background suggesting these are not partial EcoRI digestion products (compare Fig. 2B, lanes 1 and 2 to lanes 3 and 5).

Properties of pBI106 and pBI136. During the course of this investigation, it became apparent that restriction endonuclease digest patterns of the LM-resistance plasmid pBF4 were remarkably similar

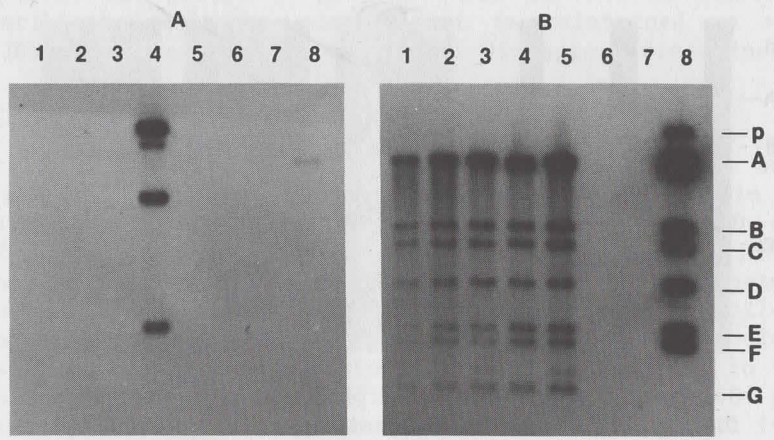


Fig. 2. Autoradigraphs of EcoRI-digested total cellular DNA from IB106 and LM resistant transconjugants following hybridization with ^{32}P-labeled pBI106 (panel A) or pBI136 (panel B). The total cellular DNAs used were as follows: IB129 (lane 1), IB128 (lane 2), IB126 (lane 3), IB106 (lane 4), IB131 (lane 5), strain 638 (lane 6), V528 (lane 7), and purified pBI136 from IB136 (lane 8). For derivation of strains see the footnotes to Tab. 2. The pBI136 EcoRI fragments (A to G) are shown at the right of the photograph. Note that an artifact in the blotting procedure prevented hybridization of the pBI136 EcoRI-G fragment to itself in Lane 8. (p), Partial digestion product.

to those of pBI106 depending upon the enzyme used (e.g., HindIII). This observation coupled with the fact that pBI136 (not pBI106), was implicated in IB106 LM resistance, prompted a comparative analysis of the 3 plasmids. Using filter blot hybridization techniques, each plasmid was used to detect homologous sequences in EcoRI digests of each of the other plasmids; the resulting autoradiographs are shown in Fig. 3. The pBI106 probe did not hybridize to pBI136 but showed strong homology to most of the pBF4 molecule. Only the 3.7 kb pBF4 EcoRI-D fragment failed to hybridize with pBI106 (Fig. 3B). This is the region that is implicated in LM resistance (24). The pBF4 probe showed homology to both pBI106 and pBI136. The hybridization to pBI106 occurred at all 3 of its EcoRI fragments but homology to pBI136 was restricted to the EcoRI-C and -G fragments (Fig. 3C). The reciprocal blots with pBI136 as the probe indicate that the cross-hybridization between pBF4 and pBI136 was primarily mediated by the pBF4 EcoRI-D fragment but some homology to the pBF4 EcoRI-B fragment was also observed (Fig. 3D). Weak homology between the pBI136 probe and DNA present in the pBI106 preparation also occurred. However, these sequences comigrated with the pBI136 EcoRI

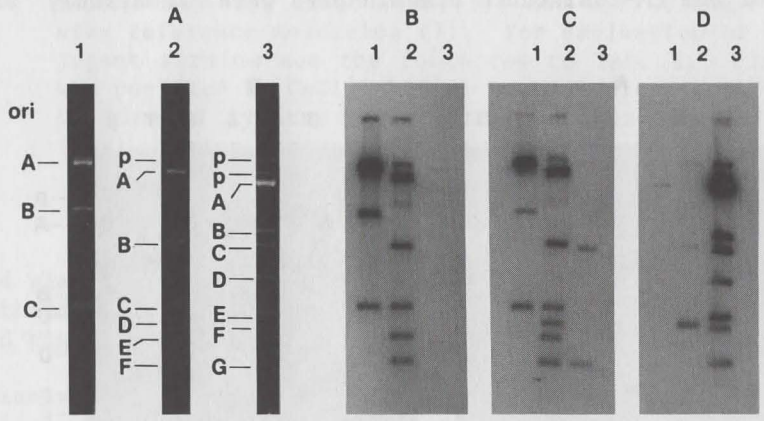


Fig. 3. Sequence homology between pBF4, pBI106, and pBI136. Purified plasmid DNA was digested with EcoRI and then samples were electrophoresed on the same agarose gel. DNA was then transferred to nitrocellulose filters (17) and then hybridized at 67°C (22) with the ^{32}P-labeled (13) plasmid DNA indicated below. Panel A, Agarose gel of digested plasmid DNAs. pBI106 (lane 1), pBF4 (lane 2), and pBI136 (lane 3). Panels B, C, and D are autoradiographs of blotted DNA from gel slices identical to panel A. The following ^{32}P-labeled probes were used: panel B pBI106, panel C pBF4, and panel D pBI136. (ori), Origin of gel; (p), partial digestion product; and the various EcoRI fragments of each plasmid are shown to the left of the appropriate gel slice in panel A.

fragments indicating that this hybridization was due to small amounts of pBI136 present in all samples of pBI106.

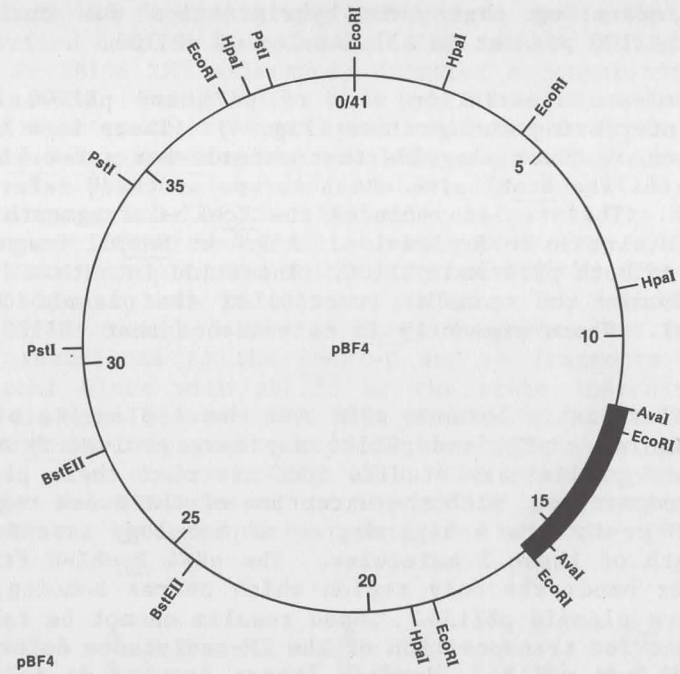
Endonuclease restriction maps of pBF4 and pBI106 revealed a number of interesting similarities (Fig. 4). There is a highly conserved region of these plasmids that extends for a few kilobases on either side of the EcoRI site which serves as the 0 reference point on the maps. This region includes the EcoRI-C fragments which are identical in size in both plasmids. A 3.4 kb BstEII fragment is also present in both pBF4 and pBI106. Insertion into this fragment of pBF4 inactivates the transfer function of the plasmid (D. Guiney, pers. comm.). There presently is no evidence that pBI106 is transmissible.

The relationship between pBF4 and the 2 plasmids of IB106 is quite intriguing. pBF4 and pBI106 may have evolved from a common ancester, and preliminary studies indicate that these plasmids are in fact incompatible. With the exception of the exact region implicated in LM resistance a high degree of homology extends over the entire length of these 2 molecules. The pBF4 EcoRI-D fragment is, on the other hand, the only region which shares homology with the LM-resistance plasmid pBI136. These results cannot be taken as direct evidence for transposition of the LM-resistance determinant between pBI106 and pBI136. However, these results do indicate that this antibiotic-resistance determinant is maintained on a highly mobile DNA sequence which is capable of widespread dissemination.

The LM-Resistance Determinant

Preliminary evidence based on homology with pBF4, suggested that the pBI136 EcoRI-C and -G fragments were involved in the LM-resistance phenotype. Use of the pBR325::pBF4 EcoRI-D chimeric plasmid pVA831 (8) as a hybridization probe against pBI136 proved that this pBF4 fragment was responsible for the homology between the 2 plasmids. The probe hybridized strongly to the pBF4 EcoRI-D fragment and to the EcoRI-C and -G fragments of 2 pBI136 molecules (Fig. 5 lanes 1, 2, and 3). In addition, the probe hybridized to the pBF4 EcoRI-B fragment. Homology to this fragment is due to a proposed directly repeated sequence present on both the -B and -D fragments (14) and will be discussed below. Previous studies (8,10,14) have shown that the pBF4 EcoRI-D fragment has homology to many of the LM-resistant Bacteroides strains including those with the nonplasmid associated determinants.

Further evidence implicating the pBI136 EcoRI-C and -G fragments in LM resistance was obtained from analysis of a spontaneously occurring, antibiotic sensitive, pBI136 deletion derivative. Restriction analysis of pBI136Δ1 showed a nearly precise deletion of the 7.2 kb EcoRI-C fragment. When this plasmid was probed with the pVA831 clone, the results confirmed that this fragment was totally absent and no fusion fragment was present, but the EcoRI-G fragment



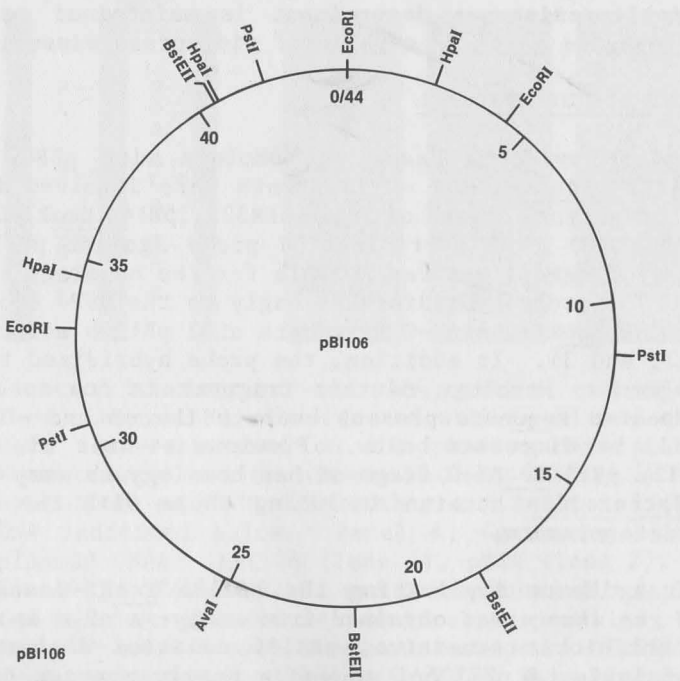


Fig. 4. Comparative endonuclease restriction site maps of pBF4 (left) and pBI106 (right). The heavy line on the pBF4 map indicates the region implicated in LM resistance.

remained intact (Fig. 5). Similar studies with pBF4 (EcoRI-D, 24) and pBFTM10 (EcoRI-B, 21) have also noted seemingly precise deletions of specific EcoRI fragments associated with the loss of LM-resistance.

Comparison of plasmid mediated determinants. Restriction endonuclease mapping (21,24) and hybridization studies (14) with pBF4 and pBFTM10 have suggested that their LM-resistance determinants are bounded on both sides by a directly repeated sequence. The repeated sequence is characterized by restriction enzyme recognition sites for EcoRI and AvaI which are separated by about 500 base pairs (bp). To further characterize the organization of the pBI136 resistance determinant, EcoRI plus AvaI double digests of pBFTM10, pBF4, and pBI136 were analyzed by filter blot hybridization using the pVA831 probe. Presumably, 500 bp of the repeated sequence is contained within the pBF4 EcoRI-D fragment of this probe. The results in Fig. 6 clearly show the presence of the 500-bp EcoRI/AvaI fragments in all 3 plasmids. The pBI136 EcoRI-C and -G fragments each have decreased in size by about 500 bp indicating that each fragment contained a copy of the repeated sequence (data not shown). As expected the pVA831 probe hybridized strongly to the pBFTM10 EcoRI-B and pBF4 EcoRI-D fragments but the probe also retained strong homology

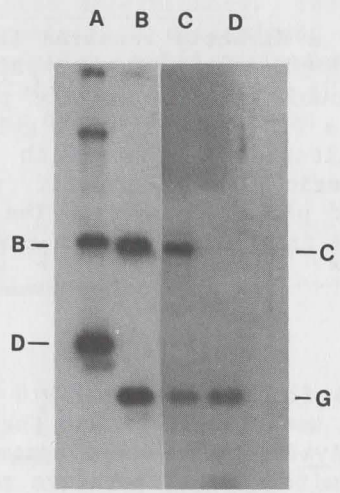


Fig. 5. Homology of pBI136 and pBI136Δ1 to the pBF4 EcoRI-D fragment cloned into pBR325. Purified plasmid DNAs were digested with EcoRI, electrophoresed on an agarose gel, blotted to nitrocellulose filters, and probed with the chimeric plasmid pVA831 (pBR325::pBF4 EcoRI-D;14). pBF4 (lane A), pBI136 from IB106 (lane B), pBI136 from IB128 (lane C), and pBI136Δ1 from IB178 (lane D). The EcoRI-B and -D fragments of pBF4 are shown to the left of the photograph and the EcoRI-C and -G fragments of pBI136 are shown to the right of the photograph.



Fig. 6. Evidence for a directly repeated sequence present on the Bacteroides R-plasmids pBFTM10, pBF4, and pBI136. EcoRI plus AvaI double digests of the plasmids were electrophoresed on a 0.8% agarose slab gel, blotted to a nitrocellulose filter, and probed with the pBF4 EcoRI-D containing chimeric plasmid, pVA831. pBFTM10 (lane A), pBF4 (lane B), and pBI136 (lane C). The common 500 bp EcoRI - AvaI fragment is indicated by an arrow to the right of the photograph.

to the EcoRI-A and -B fragments of pBFTM10 and pBF4 respectively (Fig. 6). This result would suggest that the repeated sequence extends well beyond the AvaI site in these fragments. In order to accurately define the limits of this putative repeated sequence, primary DNA sequence analysis of pBF4 has been initiated. Preliminary results from these studies have so far verified the presence of the directly repeated sequence on both the EcoRI-D and -B fragments (Rasmussen and Macrina, in prep.).

Cloning of the pBI136 EcoRI-C and -G fragments into E. coli JM83 has facilitated restriction mapping of these 2 important DNA segments. The pUC19 vector system (11) was used for standard shotgun cloning of the pBI136 EcoRI fragments, and clones containing the C, D, E, F, and G fragments were obtained. Restriction digest studies together with additional hybridization results (data not shown)

permitted the construction of the restriction map in Fig. 7. Typical of the results with pBF4 and pBFTM10, the region of pBI136 implicated in LM resistance (EcoRI-C) is flanked on both sides by restriction sites for EcoRI and AvaI in a direct orientation. The intervening DNA sequence of the pBI136 element is much larger in size than seen in pBF4 or pBFTM10 (Fig. 7). A comparison of the restriction sites within the region does not reveal any highly conserved sites except for the repeated sequence bounded by EcoRI and AvaI sites. This apparent heterogeneity makes impossible even speculative placement of the LM-resistance genes within these elements.

In addition to the LM-resistance determinant, the pBF4 EcoRI-D and pBFTM10 EcoRI-B fragments contain a cryptic tetracycline-resistance determinant (D. Guiney, pers. comm.). These determinants are expressed in aerobically grown E. coli but do not express tetracycline resistance in Bacteroides. E. coli strains containing either the pBI136 EcoRI-C or -G fragments cloned into the vector pUC19, do not express tetracycline resistance in either orientation. These results and the obvious structural differences in the 3 LM-resistance elements, imply that there has been a broad evolutionary divergence of these sequences among the Bacteroides.

Nonplasmid associated determinants. The widespread dissemination of a common LM-resistance determinant among Bacteroides includes not only the plasmid mediated resistance (14,15) but the nonplasmid associated resistance as well (5,9,10). In order to better understand the molecular organization of the nonplasmid determinant

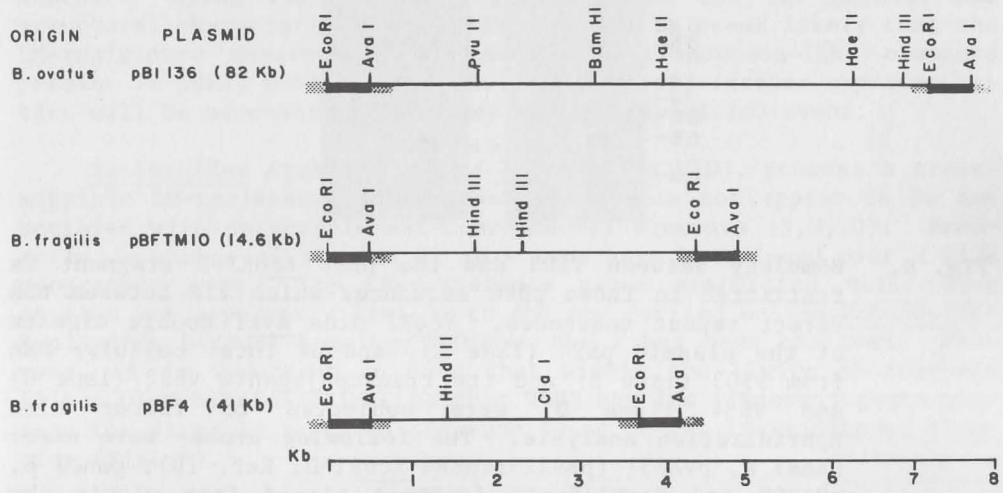


Fig. 7. Comparative endonuclease restriction maps of the LM-resistance elements from pBFTM10, pBF4, and pBI136. Data for placement of restriction sites for pBFTM10 were obtained from previously published results (14).

we have continued our studies with B. fragilis V503 (10). This previous work has shown that the pVA831 chimeric plasmid is homologous to 2 HindIII fragments present in the total cellular DNA of V503 and its LM-resistant transconjugants. Further hybridization data using the LM-sensitive deletion plasmid pBF4Δ1 (24) as a hybridization probe, failed to show hybridization with V503 (10). This plasmid probe apparently does not contain the LM-resistance determinant but does carry at least 500 bp of the directly repeated sequence (14,24). Thus these results suggested that the V503 determinant does not carry the directly repeated sequence common to the R-plasmids. Evidence supporting this conclusion was obtained by filter blot hybridization analysis of EcoRI and AvaI double digests of V503 and its transconjugants. The radiolabled probes were pVA831 or a small EcoRI/HpaII fragment from within the directly repeated sequence on the pBF4 EcoRI-D fragment. The autoradiographs in Fig. 8A show that the positive control, pBF4, hybridized strongly to the pVA831 probe at the EcoRI-B and -D fragments and at the 500 bp EcoRI/AvaI fragment. Neither V503 nor its transconjugants seem to contain the small 500-bp fragment but they do show homology with 2 fragments of about 15 kb and 6.4 kb in size. When the small pBF4 EcoRI/HpaII fragment was used as the probe (Fig. 8B), the only hybridization observed was to the positive control pBF4 in lane A. No hybridizing sequences within V503 or its transconjugants were seen even upon prolonged exposure of the autoradiograph.

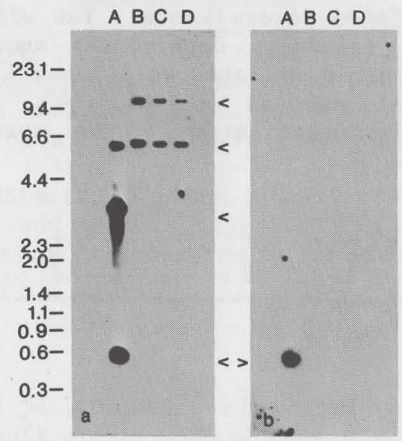


Fig. 8. Homology between V503 and the pBF4 EcoRI-D fragment is restricted to those pBF4 sequences which lie between the direct repeat sequences. EcoRI plus AvaI double digests of the plasmid pBF4 (lane A), and of total cellular DNA from V503 (lane B) and its transconjugants V622 (lane C) and V654 (lane D) were subjected to filter blot hybridization analysis. The following probes were used: Panel a, pVA831 (pBR325::pBF4 EcoRI-D, Ref. 10); panel b, pVA200 and EcoRI/HpaII fragment cloned from within the EcoRI/AvaI 500-bp fragment of pVA831. Size reference markers in kb are given to the left of the photograph and areas of hybridization are indicated by the arrows in the center of the photograph.

SUMMARY AND CONCLUSIONS

Results presented in this paper have shown that a widely distributed LM-resistance determinant is present on at least 3 distinct Bacteroides R-plasmids. In fact these plasmids bear no homology outside of the defined regions implicated in the LM resistance. This resistance determinant on pBF4, pBFTM10, and pBI136 is located within DNA segments bounded on each side by a directly repeated sequence of more than 500 bp. The intervening sequences of these 3 elements are variable, and range in size from about 3.7 kb to 7.2 kb (Fig. 7). Apart from the EcoRI/AvaI restriction sites which characterize the repeated sequence, there is a notable lack of common restriction sites within these elements. These results suggest that the elements do possess a certain degree of structural similarity but significant evolutionary divergence has occurred.

The presence and location of the directly repeated sequences, their association with specific deletions, and their association with an antibiotic-resistance determinant, are features common to many antibiotic-resistance transposons described for other prokaryotes. In addition, these elements are highly mobile, being found on a number of R-plasmids. The unique relationship between pBF4, pBI106, and pBI136 described here is a clear indication of the potential for these DNA sequences to move from one molecule to another. Given the extensive dissemination and the genetic and structural characteristics described above, it seems likely that the LM-resistance determinant is carried on transposon-like elements present in pBF4, pBFTM10, and pBI136. However, further experimentation will be necessary to document the transposition event.

Bacteroides strains such as B. fragilis V503, possess a transmissible LM-resistance determinant which does not appear to be associated with detectable extrachromosomal elements (5,9,10). Presently, a number of strains of this type have been found over a wide geographic area. The LM-resistance genes associated with these strains are apparently similar to the one carried on the Bacteroides R-plasmids because homology between the 2 has been observed. However, it is important to note that within the limits of Southern filter blot hybridization, neither V503 nor its transconjugants possess the directly repeated sequence found on the LM-resistance plasmids (Fig. 8).

The elusive nature of the V503 LM-resistance elements presents an intriguing problem. One model that has been proposed is that these resistance determinants reside on a conjugative transposon similar to Tn916 of Streptococcus faecalis (3). Such a mechanism

would account for the transmissibility and lack of extrachromosomal detection, and it would be consistent with the structural differences between the LM-resistance elements of the plasmids and that of V503. However, the recent discovery of pBI136 has provided direct evidence for the existence of difficult to detect, large molecular weight R-plasmids in the Bacteroides. Thus there is a possibility that the resistance determinants of V503 and similar strains reside on large plasmids.

Future studies with pBI136 should result in an understanding of the problems associated with its detection in many cellular backgrounds. These studies may be useful in understanding the molecular nature of the nonplasmid-associated antibiotic-resistance determinants present in Bacteroides species.

ACKNOWLEDGMENTS

This research was in part supported by Public Health Service grants DE04224 and AI/DE20153 and by a grant from the Jeffress Trust to F.L.M. We thank G. Harbaugh, Donna Bandy, and Sonja Blowe for assistance in manuscript preparation.

REFERENCES

1. Finegold, S.M. (1977) Anaerobic Bacteria in Human Disease. Academic Press, Inc., New York.
2. Finegold, S.M., H.R. Atteberg, and V.L. Sutter (1974) Effect of diet on human fecal flora: comparison of Japanese and American diets. Am. J. Clin. Nutr. 27:1456-1469.
3. Franke, A.E., and D.B. Clewell (1981) Evidence for a chromosome-borne resistance transposon (Tn916) in Streptococcus faecalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bact. 145:494-502.
4. Guerry, P., D.J. LeBlanc, and S. Falkow (1973) General method for the isolation of plasmid deoxyribonucleic acid. J. Bact. 116:1064-1066.
5. Guiney, D.G., P. Hasegawa, D. Stalker, and C.E. Davis (1983) Genetic analysis of clindamycin resistance in Bacteroides species. J. Infect. Dis. 147:551-558.
6. Holdeman, L.V., E.P. Cato, and W.E.C. Moore, eds. (1977) Anaerobe Laboratory Manual, Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, VA.
7. Macrina, F.L., D.J. Kopecko, K.R. Jones, D.J. Ayers, and S.M. McCowen (1978) A multiple plasmid-containing Escherichia coli strain: Convenient source of size reference plasmid molecules. Plasmid 1:417-420.
8. Macrina, F.L., T.D. Mays, C.J. Smith, and R.A. Welch (1981) Nonplasmid associated transfer of antibiotic resistance in Bacteroides. J. Antimicrob. Chemother. Vol. 8, Suppl. D, pp. 77-86.
9. Marsh, P.K., M.H. Malamy, M.J. Shimell, and F.P. Tally (1983) Sequence homology of clindamycin resistance determinants in clinical isolates of Bacteroides spp. Antimicrob. Agents Chemother 23:726-730.
10. Mays, T.D., C.J. Smith, R.A. Welch, C. Delfini, and F.L. Macrina (1982) Novel antibiotic resistance transfer in Bacteroides. Antimicrob. Agents Chemother. 21:110-118.
11. Norrander, J., T. Kempe, and J. Messing (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26:101-106.
12. Privitera, G., A. Dublanchet, and M. Sebald (1979) Transfer of multiple antibiotic resistance between subspecies of Bacteroides fragilis. J. Inf. Dis. 139:83-87.
13. Rigby, P.W.J., M. Diechmann, C. Rhodes, and P. Berg (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.
14. Shimell, M.J., C.J. Smith, F.P. Tally, F.L. Macrina, and M.H. Malamy (1982) Hybridization studies reveal homologies between pBF4 and pBFTM10, two clindamycin-erythromycin resistance transfer plasmids of Bacteroides fragilis. J. Bact. 152:950-953.
15. Smith, C.J., and F.L. Macrina (1984) Large transmissible clindamycin resistance plasmid in Bacteroides ovatus. J. Bact. 158:739-741.
16. Smith, C.J., R.A. Welch, and F.L. Macrina (1982) Two independent conjugal transfer systems operating in Bacteroides fragilis V479-1. J. Bact. 151:281-287.
17. Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.
18. Tally, F.P., G.J. Cuchural, N.V. Jacobus, S.L. Gorbach, K.E. Aldridge, T.J. Cloary, S.M. Finegold, G.B. Hill, P.B. Iannini, R.V. McCloskey, J.P. O'Keefe, and C.L. Pierson (1983) Susceptibility of the Bacteroides fragilis group in the United States in 1981. Antimicrob. Agents Chemother. 23:536-540.
19. Tally, F.P., M.J. Shimell, G.R. Carson, and M.H. Malamy (1981) Chromosomal and plasmid mediated transfer of clindamycin resistance in Bacteroides fragilis. In Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids, S. Levy, R.C. Clowes, and E. Koenig, eds. Plenum Press, New York, pp. 51-59.
20. Tally, F.P., D.R. Snydman, S.L. Gorbach, and M.H. Malamy (1979) Plasmid mediated transferable resistance to clindamycin and erythromycin in Bacteroides fragilis. J. Infect. Dis. 139:83-88.
21. Tally, F.P., D.R. Snydman, M.J. Shimell, and M.H. Malamy (1982) Characterization of pBFTM10, a clindamycin-erythromycin resistance transfer factor from Bacteroides fragilis. J. Bact. 151:686-691.11.

22. Thayer, R.E. (1980) An improved method for detecting foreign DNA in plasmids of Escherichia coli. Anl. Biochem. 98:60-63.
23. Welch, R.A., K.R. Jones, and F.L. Macrina (1979) Transferable lincosamide-macrolide resistance in Bacteroides. Plasmid 2:261-268.
24. Welch, R.A., and F.L. Macrina (1981) Physical characterization of the Bacteroides R plasmid pBF4. J. Bact. 145:867-872.

EFFECT OF CHROMOSOME HOMOLOGY OF PLASMID TRANSFORMATION AND PLASMID CONJUGAL TRANSFER IN HAEMOPHILUS INFLUENZAE

M. Balganesh and J. K. Setlow

Biology Department
Brookhaven National Laboratory
Upton, New York 11973

INTRODUCTION

Homology between a resident plasmid in H. influenzae and a plasmid entering the cell has been shown to be very important for the acquisition by the cell of genetic markers from the incoming plasmid. For example, the frequency of transformation of recombination-proficient H. influenzae by two 51-kbp plasmids increases by more than 3 orders of magnitude when the recipient cells contain a partially homologous plasmid (1). This increase is not simply the result of increased uptake of plasmid DNA into the cell, since no transformation could be observed with Rec^- recipients which take up DNA as well as the wild type. Furthermore, one of the plasmids has been shown to contain the DNA uptake sites necessary for efficient uptake into competent H. influenzae cells (Setlow and Albritton, unpublished experiments), and the data suggest that the other plasmid also contains such sites. Thus, the plasmids are able to transform Rec^+ cells containing homologous DNA at a higher frequency because of events occurring following DNA uptake.

A study of transformation by a hybrid plasmid, pNov1, constructed by inserting a 9 kbp piece of chromosomal DNA coding for resistance to novobiocin into the 5.7-kpb plasmid RSF0885, showed that there is a similar effect of homology on the establishment of this plasmid (16). Efficient transformation by pNov1 is also dependent on the Rec^+ genes of the recipient. There is considerable recombination between homologous regions of the plasmid pNov1 and the chromosome in Rec^+ recipients, resulting in around 75% substitution of homologous chromosomal genetic information for plasmid genes in

the established plasmid. Even more probable than plasmid establishment following uptake is the integration of homologous parts of the plasmids into the chromosome, causing loss of the plasmid from the cell. This interaction takes place at the time of the transformation rather than after the plasmid becomes established (16).

Competence in H. influenzae is a physiological state in which DNA may be taken up efficiently from the medium (7). However, the competent state involves more than ability to take up DNA. There is also a competence-induced increase in the ability of DNA to recombine. For example, the frequency of H. influenzae phage recombination is around 10 times higher in competent cells than in exponentially growing or stationary phase cells (4). The chromosomal DNA of competent cells contains regions of single-strand gaps and tails (10,11). These regions are apparently necessary for recombination between transforming DNA and the recipient chromosome. A recombination-defective mutant, rec2, that is transformed with a frequency 10^{-7} that of the wild type, does not contain such regions, although when it is in the competent state it takes up DNA from the medium as well as does the competent wild type. A possible explanation of the interaction between plasmids and chromosomes during transformation, but not after the plasmids become established, was that the competent state was required for such interaction. However, no interaction was observed when wild-type cells with an established plasmid were made competent (16). Thus, the interaction apparently only takes place upon entrance of the plasmid into the cell. We present evidence here that when a plasmid enters the cell by a route other than transformation, namely conjugal transfer, there is also no interaction between homologous regions of plasmid and chromosome. We also show that the ability of a plasmid to become established by transformation is dependent upon the degree of homology between plasmid and chromosome, but such homology does not increase plasmid establishment following conjugal transfer.

METHODS

In order to make an adequate comparison of the effect of chromosomal homology on transformation and conjugal transfer, we needed plasmids with and without homology that could both transform and be transconjugated. Since the smaller plasmids in H. influenzae are not conjugally transferred, we used the 35-kbp mobilizing plasmid pHD147 (6). Unfortunately, we could not detect mobilization of pNov1 (Tab. 1), although the parent plasmid RSF0885 was mobilized at a reasonable frequency, as previously reported (6). These data suggest that the PvuII site in RSF0885, used for insertion of the chromosomal DNA of pNov1, is involved with mobilizability. Therefore, we have used another cloning vehicle, pDM2, previously constructed in this laboratory (12), and have ligated into it chromosomal portions from pNov1. Figure 1 shows a simplified restriction

Tab. 1. Mobilization of RSF0885 and pNov1.

Mating pairs	Transconjugation frequency
rec1(pNov1)(pHD147) x rec2	$<8 \times 10^{6}$
rec1(pNov1)(pHD147) x wild type	$<8 \times 10^{-7}$
rec1(RSF0885)(pHD147) x rec2	10^{-3}
rec1(RSF0885)(pHD147) x wild type	10^{-3}

Cells were mixed and incubated overnight on hemoglobin agar, a condition that fosters conjugal transfer of plasmids (Albritton, Setlow and Slaney, 1982). The recipients (rec2 or wild type) were resistant to streptomycin, and RSF0885 and pNov1 in the donors conferred resistance to ampicillin. The transconjugation frequency was measured as the ratio of viable cells doubly resistant to ampicillin and streptomycin to the total number of recipients (the streptomycin-resistant cells).

map of pNov1 as well as drawings of the other plasmids used. None of these plasmids contains an active transposon. The 9.8-kbp parent plasmid pDM2, coding for ampicillin and chloramphenicol resistance, contains 2 EcoRI sites separated by 1.1 kbp. Following digestion by EcoRI, the large (8.7-kbp) fragment was then ligated together with itself, or in the case of pMB1 and pMB2, ligated to 1 (pMB1) or 2 (pMB2) copies of the 1.7 kbp EcoRI fragment of pNov1. The large plasmid pMB3 was constructed by first digesting the protruding single-strand ends of EcoRI-cut pDM2s with S1 nuclease, and then blunt-end ligating the whole of the chromosomal portion of pNov1, removed by PvuII digestion. The SmaI-cut vector was also blunt-end ligated to the 9.0-kbp pNov1 fragment. The latter plasmid, pMB4, no longer coded for chloramphenicol resistance. The other plasmids, however, conferred both ampicillin and chloramphenicol resistance. The 1.7-kbp EcoRI fragment of pNov1 confers resistance to 25 µg/ml novobiocin, but is not expressed in cells carrying pMB1 or pMB2, unless the resistance gene has been transferred to the chromosome. In cells carrying pMB3 or pMB4, which include from pNov1 the 1.7-kbp fragment as well as chromosomal DNA on either side of that fragment, the novobiocin resistance is expressed even without recombination with the chromosome. Figure 2 shows evidence that the orientation of the single EcoRI fragment in pMB1 is the same as that of the 2 EcoRI fragments in pMB2.

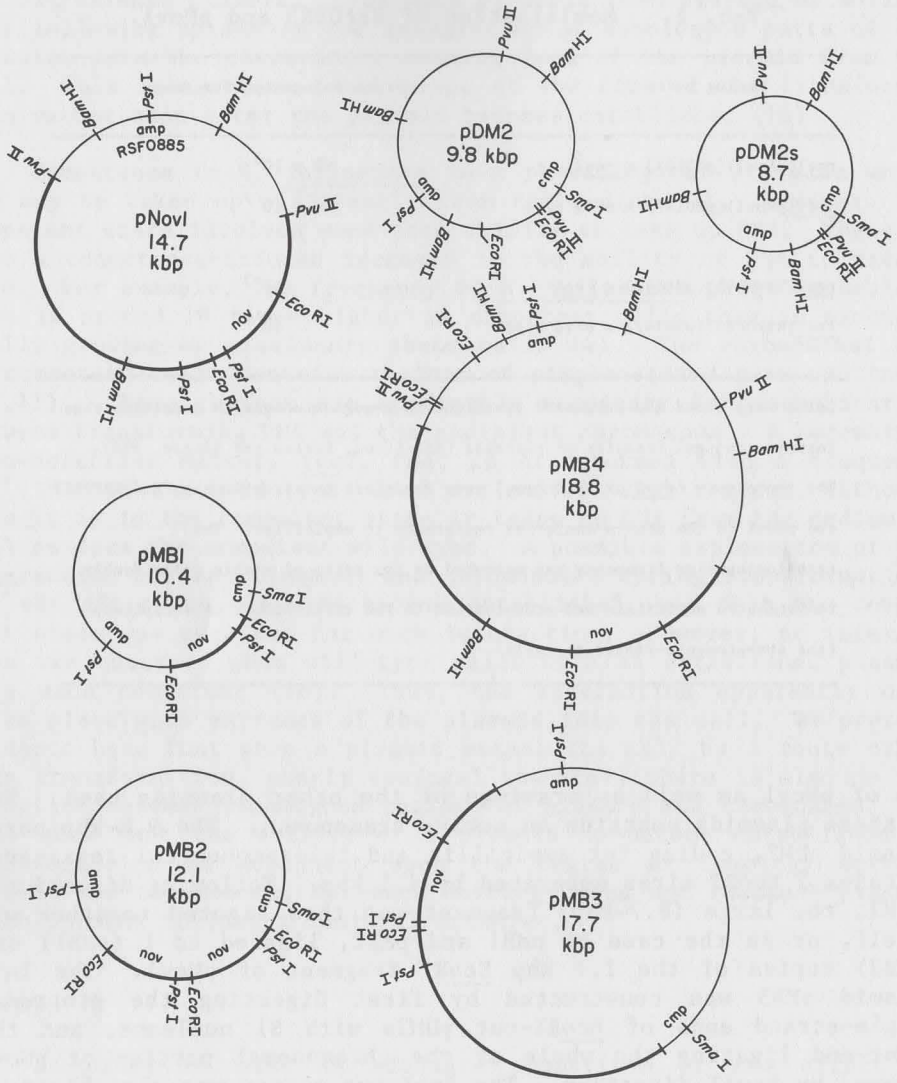


Fig. 1. Plasmids used in this study.

Transformation was carried out as previously described (15) in cells made competent in MIV medium (17). All of the plasmids we have constructed, except pNov1, were mobilizable by pHD147 in three-way matings. Mating was usually done on membrane filters on top of hemoglobin agar for 2 to 3 hr (with little or no observable difference in transconjugation frequency between these times). Because of the phenomenon of Rec^+-dependent Rec^- transfer of chromosomal markers from cell to cell (2), we used Rec^- strains for the donors, to avoid the possibility that chromosomal markers in the recipients could enter donor cells and give spurious numbers for transconjugant frequency. Transconjugation was completely dependent on the presence of the mobilizing plasmid, as shown in the example of Tab. 2. The frequencies were independent of the recombination proficiency of the recipient, as in the two-way mating shown in Tab. 1, and were also independent of the state of competency of the recipient, and whether deoxyribonuclease at 125 µg/ml was added to the cells at the time of mixing them and adding them to the filter (data not shown).



Fig. 2. Gel electrophoresis of pMB1 (b) and pMB2 (c) cut with PstI, along with molecular weight standards (HpaI digested T7 DNA) (a). The numbers refer to kbp. Since the digestion of pMB1 yields 2 fragments the same size as 2 of the 3 fragments of digested pMB2, the orientation of the 2 EcoRI chromosomal fragments in pMB2 must be the same as the orientation of the single chromosomal fragment of pMB1.

RESULTS

Transformation and Transconjugation Frequencies

Figure 3 shows a comparison of frequency of plasmid establishment following transformation or conjugation as a function of size of the chromosomal insert in wild-type or Rec^- recipients. The plasmid pDM2s with its EcoRI piece removed is more comparable to the larger plasmids than the untreated pDM2. Conjugal transfer but not

Tab. 2. A typical mating experiment.

Mating cells	Total Number of Cells in the Suspension: Donors	Containing the mobilizing plasmid	Recipients	Transconjugants
rec2(pHD147) recl(pMB3) Rd(wild type)	1.6×10^9	2.4×10^9	1.2×10^9	1.2×10^5
recl(pMB3) Rd	2.2×10^9	-	8.8×10^8	0 (<1 in 4 x 10^6 recipients)

After incubation of the cells on a filter for two hours, they were suspended in a volume of 2 ml, and plated with appropriate dilutions and antibiotics. The donors, recl(pMB3), were resistant to novobiocin and chloramphenicol, the cells containing the mobilizing plasmid, rec2(pHD147), were resistant to kanamycin, and the recipients, Rd, were resistant to streptomycin. Transconjugants were resistant to both streptomycin and chloramphenicol.

transformation was depressed in pDM2s relative to that of pDM2. There was an exponential increase in the number of wild-type transformants with increasing size. The data for transformation of the Rec⁻ strain show that even the smallest insert increased the transformation several orders of magnitude over that of pDM2 without an insert. This increase is presumably the result of the presence of uptake sites on the inserts. However, the larger inserts did not cause a higher frequency of transformation in the Rec⁻ cells; on the contrary, the frequency decreased slightly with an increase in insert size. The transformation of the rec2 strain by the plasmids with inserts was much lower than in the wild-type. Similar results were obtained with recl recipients (data not shown).

The conjugal transfer results were not different for the wild-type and Rec⁻ recipients, and there was relatively little effect of insert size, except that there was a decrease in frequency of transfer of the largest plasmid.

Figure 4 shows transformation to novobiocin resistance in the same experiment presented in Fig. 3. The data for plasmid establishment (transformation to chloramphenicol resistance) from Fig. 3 are also shown for comparison. The plasmid with 2 inserts of the novobiocin resistance marker, pMB2, did not transform to novobiocin resistance as well as the plasmid with only 1, pMB1, although pMB2 became established with a frequency around 5 times higher. However, the transformation to novobiocin resistance by the largest plasmid was considerably higher than by the other 2 plasmids.

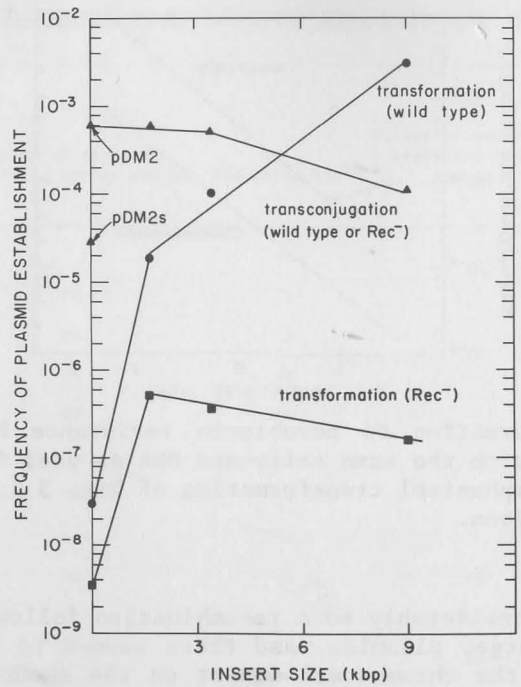


Fig. 3. Frequency of plasmid establishment by transformation and transconjugation as a function of size of the chromosomal insert in the EcoRI site of pDM2. Final concentration of purified plasmid DNA for transformation was 0.3 µg/ml. Transformation by pDM2 and pDM2s yielded the same frequency.

Recombination Between Plasmids and Chromosomes

Table 3 shows measurements of plasmid-chromosome recombination in transformants and transconjugants. Transformants or transconjugants containing pMB3 or pMB4, were selected by resistance to chloramphenicol or ampicillin. They were picked, grown up in chloramphenicol or ampicillin (the parent plasmid markers) and spotted on plates containing parent plasmid marker or this marker plus novobiocin. A clone was considered to be a recombinant if it had become novobiocin-sensitive. Since the novobiocin-resistance markers of pMB1 and pMB2 are not expressed unless they are integrated into the chromosome, we tested for transfer of novobiocin sensitivity from chromosome to plasmid by determining transformation to novobiocin resistance from lysates of chloramphenicol transformants of these plasmids. When a lysate failed to show any evidence of novobiocin resistance markers, the plasmids must have acquired novobiocin sensitivity from the chromosome.

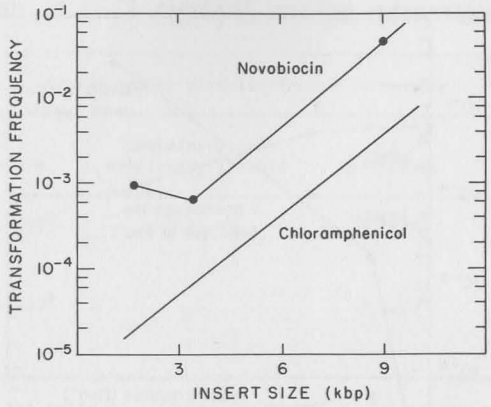


Fig. 4. Transformation to novobiocin resistance by pDM2 derivatives with the same cells and DNA as used for Fig. 3. The chloramphenicol transformation of Fig. 3 is also shown for comparison.

There was considerably more recombination following transformation with the larger plasmids, and there seemed to be an effect of the position of the chromosomal insert on the amount of recombination. While the inserts were the same size in pMB3 and pMB4, there was about twice as much recombination when the insert was in the SmaI site (pMB4) as when it was in the EcoRI site (pMB3). The difference between the observed recombination in pMB1 and pMB2 may not be significant. However, there was clearly less recombination in these than in the larger plasmids, indicating that decrease in size of the homologous region results in decrease in plasmid-chromosome interaction resulting in recombination.

Table 3 also shows that while all the plasmids interacted with the chromosome in transformation, there was no recombination observed among transconjugants, even when the recipient was competent. Following mating, novobiocin-resistant recipient colonies were picked, grown in novobiocin, and spotted on plates containing novobiocin or novobiocin plus chloramphenicol or ampicillin. The fact that all the clones tested were resistant to both drugs indicated that there had been no integration of the novobiocin marker into the chromosome, a procedure that would be expected to cause loss of the plasmid and the parent plasmid resistance marker. In accord with this conclusion was the fact that when some of the pMB4-containing transconjugants were treated so as to lose the plasmid, in all 18 cured clones tested both novobiocin and ampicillin resistance had been lost.

Further evidence for lack of interaction between conjugally transferred plasmids and chromosome is that we failed to find any novobiocin-resistant recipient cells following conjugation that resulted in transfer of pMB1 or pMB2. Although these plasmids do not express the novobiocin marker, the marker is expressed when there is recombination resulting in transfer of the resistance gene into the chromosome. The results of Tab. 4 show that there was no such recombination detectable, even though the wild type (strain Rd) was used as recipient.

Tab. 3. Recombination between plasmids and chromosomes.

Plasmid	Number of plasmid-containing clones tested	% Recombination[a]	Number of novobiocin resistant clones tested	Number of ampicillin or chloramphenicol sensitive clones[b]
Transformation				
pMB1	20	15		
pMB2	20	5		
pMB3	84	38		
pMB4	83	71		
Transconjugation				
pMB4 (noncompetent recipient)	82	0	61	0
pMB4 (competent recipient)	48	0	44	0
pMB3 (noncompetent recipient)			60	0

[a] % of novobiocin-sensitive plasmid-containing clones (for pMB1 and pMB2 this was the % of clones not containing DNA capable of transformation to novobiocin resistance).

[b] among the novobiocin-resistant clones.

The recipients in the matings were all wild type and originally streptomycin-resistant and novobiocin-sensitive. Donors and mobilizing plasmid strains were Rec$^-$ and streptomycin-sensitive.

DISCUSSION

The data of Fig. 4 suggest that the pairing between the plasmid and homologous parts of the chromosome that increases plasmid establishment may not be the same as the pairing that results in integra-

Tab. 4. Mating involving plasmids which do not express the novobiocin-resistance marker unless there is integration of plasmid DNA into the chromosome.

Mating cells	Number of cells/ml of suspension: Donors	Recipients	Recipients containing plasmids	Novobiocin-resistant recipients
rec2(pHD147) recl(pMB1) Rd	$4x10^8$	$3x10^8$	$5x10^5$	0 (<1 in $3x10^7$ recipients
rec2(pHD147) recl(pMB2) Rd	$6x10^8$	$5x10^8$	$6x10^5$	0 (< in $5x10^7$ recipients

The cells were mixed and incubated overnight on hemoglobin agar. Some were then scraped off, resuspended and plated, after appropriate dilution, with antibiotics to distinguish between the strains. Resistance of the strains was the same as in Table 2, except that recl (pMB1) and recl(pMB2) were resistant to chloramphenicol only.

tion of a homologous region of the plasmid into the chromosome. Thus, novobiocin transformation decreases with the duplication of the chromosomal portion in pMB2, but establishment of the plasmid increases with this duplication. A model to explain these data is shown in Fig. 5. The homologous region of the plasmids is labeled A B C, to be paired with A'B'C' of the chromosome. The smaller plasmid, pMB1, is likely to pair with the chromosome in a contiguous manner. However, the pairing of the larger plasmid could also involve 1 portion of each of the 2 inserts, with a looped portion between. The model suggests that transformants of pMB2 should sometimes have lost the second copy of the EcoRI fragment, and thus be converted to pMB1, because of the possibility of elimination of the unpaired homologous region. Twenty chloramphenicol transformants of pMB2 were picked, grown up and cleared lysates made and run on a gel. Sixteen of them were found to contain plasmids the size of pMB1, and 4 contained plasmids of the original size of pMB2. These data are in accord with the model.

Recently, a model has been proposed for transformation in H. influenzae (3), in which DNA is first taken up into extensions of the membrane on the cell surface, which have been called "transformasomes." Following uptake of a linear DNA and its partial degradation, a single strand of the DNA can enter the cytoplasm of the cell. This single strand can then pair with a homologous single-strand region of the recipient cell chromosome, leading to integration. There is some evidence for this model. The membrane extensions have been visualized, and shown to be more prevalent on competent cells (5, 9). A pore has been seen between the transformasome and the cytoplasm, through which the single strand of DNA could pass (9). Covalently closed circles do not readily exit from the transformasomes, nor do molecules with hairpin ends, and both of these are protected from degradation in the transformasome (3,8). Linear DNA, however, is degraded so that it has a single-stranded end (3,14). The DNA of competent H. influenzae has been shown to have one strand containing gaps and tails (10,11) that are necessary for pairing between transforming DNA and recipient DNA. Finally, it has been known for a long time that at least the overwhelming majority of the transforming DNA is integrated as a single strand in the double-stranded recipient molecule (13).

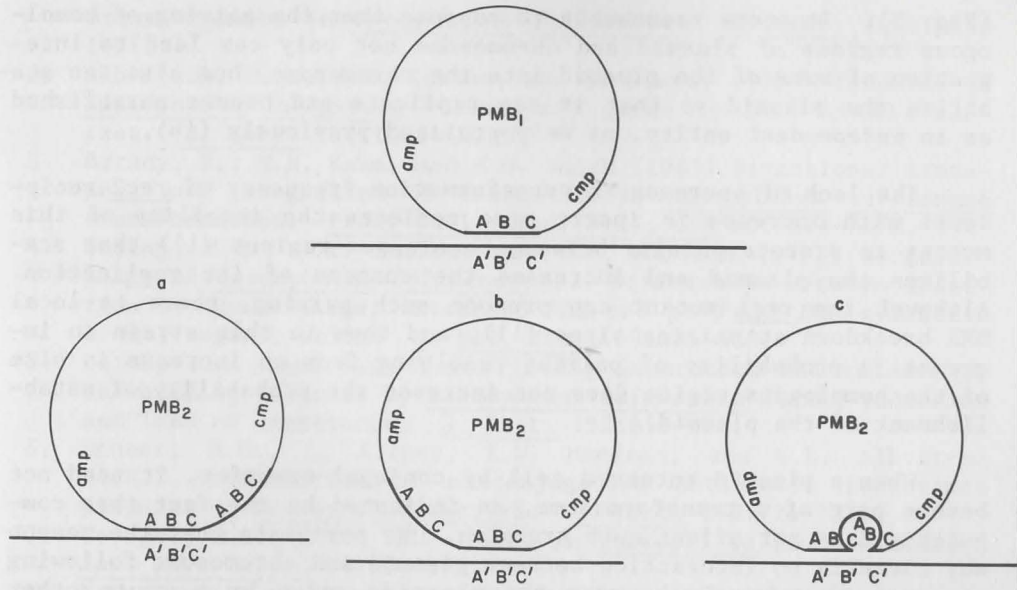


Fig. 5. A model for pairing of homologous parts of pMB1 and pMB2 with the recipient chromosome.

It is of interest to consider how the present results may be interpreted in terms of the model. The consideration is why the plasmids we have used transform at all. The cells were exposed to closed circular, supercoiled DNA (the form isolated from CsCl gradients). Since open circular or linear molecules are the only ones that exit rapidly from the transformasomes (3), either our plasmids become nicked inside the cell or their entrance into the cytoplasm takes a very long time.

We may also ask why the size of the homologous insert in the plasmids has such an enormous effect on the transformation frequency

(Fig. 3). It seems reasonable to suppose that the pairing of homologous regions of plasmid and chromosome not only can lead to integration of some of the plasmid into the chromosome, but also can stabilize the plasmid so that it can replicate and become established as an independent entity, as we postulated previously (16).

The lack of increase in transformation frequency of rec2 recipients with increase in insert size reflects the inability of this mutant to promote pairing between homologous regions (11) that stabilizes the plasmid and increases the chances of its replication. Although the rec1 mutant can promote such pairing, there is local DNA breakdown at pairing sites (11), and thus in this strain an increase in probability of pairing resulting from an increase in size of the homologous region does not increase the probability of establishment of the plasmid.

When a plasmid enters a cell by conjugal transfer, it need not become part of a transformasome, as indicated by the fact that competence does not affect such transfer. We postulate that the reason why there is no interaction between plasmid and chromosome following conjugal transfer is because the plasmids enter by a route other than transformation. The interaction may require single-strand ends to be formed, and this process may only take place when DNA is to go through the transformasome pore into the cytoplasm. The single-strand ends in the DNA of recipient cells alone do not result in interaction between plasmid and chromosome, since there was little or no interaction upon conjugal transfer of plasmids into competent cells (Tab. 3), and we observed earlier that when cells containing an established plasmid, pNov1, were made competent, there was no plasmid-chromosome interaction (16).

Antibiotic resistance in H. influenzae is undoubtedly spread in nature by conjugal transfer of plasmids. Our present observations suggest that at least in the absence of transposons there is rarely or never exchange of antibiotic resistance markers between plasmid and chromosome following such transfer.

ACKNOWLEDGMENTS

We thank William L. Albritton for the gift of the mobilizing plasmid, pHD147, and for help with the experiment showing lack of mobilization of pNov1. Research was carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy.

REFERENCES

1. Albritton, W.L., J.W. Bendler, and J.K. Setlow (1981) Plasmid transformation in Haemophilus influenzae. J. Bact. 145:1099-1101.
2. Albritton, W.L., J.K. Setlow, and L. Slaney (1982) Transfer of Haemophilus influenzae chromosomal genes by cell-to-cell contact. J. Bact. 152:1066-1070.
3. Barany, F., M.E. Kahn, and H.O. Smith (1983) Directional transport and integration of donor DNA in Haemophilus influenzae transformation. Proc. Natl. Acad. Sci., USA 80:7274-7278.
4. Boling, M.E., J.K. Setlow, and D.P. Allison (1972) Bacteriophage of Haemophilus influenzae I. Differences between infection by whole phage, extracted DNA and prophage DNA extracted from lysogenic cells. J. Mol. Biol. 63:335-348.
5. Deich, R.A., and L.C. Hoyer (1982) Generation and release of DNA-binding vesicles by Haemophilus influenzae during induction and loss of competence. J. Bact. 152:855-864.
6. Deneer, H.G., L. Slaney, I.W. Maclean, and W.L. Albritton (1982) Mobilization of nonconjugative antibiotic resistance plasmids in Haemophilus ducreyi. J. Bact. 149:726-732.
7. Goodgal, S.H. (1982) DNA uptake in Haemophilus transformation. Ann. Rev. Genet. 16:169-192.
8. Kahn, M.E., F. Barany, and H.O. Smith (1983) Transformasomes: Specialized membranous structures that protect DNA during Haemophilus transformation. Proc. Natl. Acad. Sci., USA 80:6927-6931.
9. Kahn, M.E., G. Maul, and S.H. Goodgal (1982) Possible mechanism for donor DNA binding and transport in Haemophilus. Proc. Natl. Acad. Sci., USA 79:6370-6374.
10. LeClerc, J.E., and J.K. Setlow (1975) Single-strand regions in the deoxyribonucleic acid of competent Haemophilus influenzae. J. Bact. 122:1091-1102.
11. LeClerc, J.E., and J.K. Setlow (1975) Transformation in Haemophilus influenzae. In Mechanisms in Recombination, R.F. Grell, ed. Plenum Press, New York, pp. 187-207.
12. McCarthy, D., N.L. Clayton, and J.K. Setlow (1982) A plasmid cloning vehicle for Haemophilus influenzae and Escherichia coli. J. Bact. 151-1605-1607.
13. Notani, N., and S.H. Goodgal (1966) On the nature of recombinants formed during transformation in Haemophilus influenzae. J. Gen. Physiol. 49 (Part 2):197-209.
14. Sedgwick, B., and J.K. Setlow (1976) Single-stranded regions in transforming deoxyribonucleic acid after uptake by competent Haemophilus influenzae. J. Bact. 125:588-594.
15. Setlow, J.K., D.C. Brown, M.E. Boling, A. Mattingly, and M.P. Gordon (1968) Repair of deoxyribonucleic acid in Haemophilus influenzae. I. X-ray sensitivity of ultraviolet-sensitive mutants and their behavior as hosts to ultraviolet-irradiated bacteriophage and transforming deoxyribonucleic acid. J. Bact. 95:546-558.
16. Setlow, J.K., N.K. Notani, D. McCarthy and N.-L. Clayton (1981) Transformation of Haemophilus plasmid RSF0885 containing a

cloned segment of chromosomal deoxyribonucleic acid. J. Bact. 148:804-811.
17. Steinhart, W.L., and R.M. Herriott (1968) Fate of recipient deoxyribonucleic acid during transformation in Haemophilus influenzae. J. Bact. 96:1718-1724.

PLASMID DNA PRIMASES AND THEIR ROLE IN BACTERIAL CONJUGATION

B.M. Wilkins, L.K. Chatfield, C.C. Wymbs,
and A. Merryweather

Department of Genetics
University of Leicester
Leicester LE1 7RH, England

INTRODUCTION

RNA primers necessary for starting DNA chain growth are generally made by the transcriptional activity of a group of enzymes called DNA primases. The primase of Escherichia coli is the 60,000 dalton (60 kd) product of the dnaG gene, which functions to generate primers for discontinuous replication of the bacterial chromosome and of at least some plasmids, as well as for complementary strand-synthesis on certain single-stranded genomes (24). Recently a variety of conjugative plasmids have been shown to encode DNA primases active in an assay requiring primer synthesis on single-stranded phage fd DNA. The list includes members of the B, C, I_1 (= Iα), I_1+B, I_2, K, M, P, and U incompatibility groups (44). Representative plasmids of most of the other groups, including F, have been screened in this assay and are currently classified as primase negative (26), but it is possible that some may specify DNA primases that are unable to use fd DNA as a template. In this article, which concentrates on the primases specified by ColIb-P9 ($IncI_1$), RP4 (IncP), and R16 (IncB), we review the biochemistry of these enzymes and the organization of the appropriate structural genes, and discuss the physiological role of their products in plasmid DNA metabolism during bacterial conjugation.

BIOCHEMISTRY OF PLASMID DNA PRIMASES

A Perspective of E. Coli Primer-Generating Systems

The biochemistry of plasmid DNA primases needs to be considered in context with E. coli primer-generating systems. Three such bacterial mechanisms have been defined through in vitro studies of

complementary strand-synthesis on single-stranded phage genomes, coated with bacterial single-stranded DNA binding protein (SSB). This area has been reviewed recently by Kornberg (24,25) and Nossal (33). SSB is required for bacterial DNA replication, and it acts in these in vitro systems to direct initiation and promote chain elongation by DNA polymerase III holoenzyme, the primary replicative DNA polymerase of E. coli.

The simplest system involving dnaG protein works with phage G4 DNA; the enzyme recognizes a region containing 2 potential hairpin structures and synthesizes, in a rifampin-resistant process, a specific RNA primer of 26 to 29 nucleotides. In a reaction coupled to DNA synthesis, primers are shorter and contain deoxyribonucleotides as well as ribonucleotides.

A more complex system, thought to reflect primer generation during the replication of some plasmids (22,32,39,45) and of bacterial DNA, has been defined using SSB-coated ϕX174 DNA. This process requires at least 6 prepriming proteins (dnaB and dnaC proteins, together with proteins i, n, n' and n") to allow primer generation by dnaG primase. These proteins are required for assembly and function of a multiprotein complex, termed the primosome, that is inferred to move progressively in the 5' to 3' direction from its assembly site to allow synthesis of multiple primers. The dnaB protein in this complex is envisaged to alter the configuration of single-stranded DNA so that it may be utilized by dnaG protein for primer synthesis.

The third primer-generating system is sensitive to rifampin and involves RNA polymerase itself: the enzyme recognizes a region rich in potential secondary structure within SSB-coated DNA of phages fd and M13, and synthesizes a specific RNA primer.

$IncI_1$ Plasmid Primase

The first plasmid-specified DNA primase to be purified and characterized was the encoded by R64drd-11 (29). Since other plasmids of this group, such as ColIb-P9drd-1 and R144drd-3, apparently carry homologous primase genes (15,16), the properties of the enzymes will be considered as a group. The $IncI_1$ plasmid primase gene (sog) specifies 2 antigenically related polypeptides with apparent molecular masses of 240 and 180 kd but only part of the larger protein has primase activity (see a later section). While R64drd-11 primase activity was originally found to be associated with a 140 kd polypeptide (29), a protein of this size is undetectable in crude cell extracts using immunoassay, and it may have been selected during purification as a degradation product of a larger molecule (E. Lanka, pers. comm.). $IncI_1$ plasmid primase has a remarkable ability to generate primers on a range of different single-stranded phage genomes in vitro, since it can substitute for RNA polymerase, dnaG protein, and at least the dnaB-, dnaC-, and

dnaG- protein components of the primosome in synthesizing functional primers on the DNA of phages fd, G4, and ϕX174, respectively. The enzyme generates short RNA primers in a rifampin-resistant process and these are elongated specifically by DNA polymerase III holoenzyme (29). Primers made in vitro range in size from 2 to about 10 nucleotides and, unlike primers made by dnaG protein, which contain a purine in the initiating nucleotide (3,34), they begin with cytidine-5'-monophosphate or cytidine with AMP in second position (26a).

In vivo, $IncI_1$ plasmid primase can apparently substitute for mutant dnaG protein during replication of the bacterial chromosome and other replicons, including $IncI_1$ and ColE1-like plasmids and phage λ DNA (42). This substitution was originally inferred from the ability of $IncI_1$ plasmids to suppress the temperature- sensitivity of DNA replication in dnaG mutants of E. coli (41). The plasmid gene responsible was designated sog (suppression of dnaG; Ref. 9) and shown through DNA cloning experiments to be the structural gene for the DNA primase (42).

Efficient suppression of the dnaG3 mutation by an $IncI_1$ plasmid requires derepression of the conjugative-transfer genes, suggesting that the primase functions during bacterial conjugation (41). Suppression has also been reported to require recA protein (35), but any involvement of this protein in the process must be indirect because a recombinant plasmid (pLG214; Ref. 42) specifying a truncated sog primase of 87 kd allowed dnaG3 recA1 cells to form colonies at 40°C with almost 100% efficiency (J.J. Overell and B.M. Wilkins, unpub. data). Since ColIb-P9drd-1, which carries a mutation derepressing expression of transfer genes, failed to suppress the temperature-sensitivity of DNA replication in dnaB70 mutants at 42°C, and in dnaC1 and dnaC2 strains at 41°C (unpub. data, and see Fig. 2 in Ref. 7), sog primase apparently cooperates in bacterial DNA replication with at least some primosome proteins. This contrasts the ability of the enzyme to generate primers on ϕX174 DNA in dnaB and dnaC cell extracts, and could reflect involvement of primosome proteins in movement of the replication fork along double-stranded DNA.

RP4 Plasmid Primase

The DNA primase specified by RP4 is the product of a gene designated pri and, like sog primase, it substitutes for the normal bacterial primer-generating mechanisms in initiating complementary strand-synthesis on the genomes of phages fd, G4, and ϕX174 (26). RP4 suppresses the dnaG3 mutation weakly but Pri^+ recombinant plasmids containing a mutation(s) that causes overproduction of the enzyme and a change in its specific activity allow dnaG3 cells to form colonies at 40°C with 100% efficiency (28). The pri gene specifies 2 antigenically related polypeptides of 118 and 80 kd, both of which possess primase activity (see a later section). The

RP4 enzyme synthesizes primers of similar composition and size distribution to those made by ColIb-P9 primase (26a), but the 2 enzymes are immunologically unrelated (26). Furthermore, sog and pri are not homologous since a fragment of ColIb-P9 containing sog did not hybridize with RP4 (14).

R16 Plasmid Primase

While the primase specified by R16, an IncB plasmid, has been less extensively characterized than the primases of $IncI_1$ plasmids and RP4, current evidence suggests that all 3 enzymes are functionally similar. The R16 enzyme initiates DNA synthesis on viral strands of phage M13 in cell-free extracts containing rifampin, and it promotes bacterial DNA replication in dnaG3 cells at 40°C. The gene, called pri, is different from sog and the RP4 pri determinant since a cloned primase-specifying fragment of R16 did not hybridize with RP4 or $IncI_1$ plasmids. Moreover, the R16 enzyme is immunologically distinct from sog primase and it has a larger apparent molecular weight (14,15).

ORGANIZATION OF PLASMID PRIMASE GENES

The $IncI_1$ plasmid sog gene and RP4 pri gene have been cloned and analysis of recombinant plasmids indicates that each locus specifies synthesis of 2 polypeptides that are apparently separate in-phase translation products.

The sog gene is located in an 8.1 kilobase pair (kb) EcoRI restriction fragment of ColIb-P9drd-1 (E-3 according to the numbering system of Uemura and Mizobuchi, Ref. 38) and this was cloned in pBR325 to give recombinant plasmid pLG215 (Fig. 1). The DNA fragment specifies 2 polypeptides, of 240 and 180 kd, which are also detectable in extracts of cells harboring the ColIb-P9 plasmid. The genetic determinants of these proteins overlap, since both are truncated by deletions in the cloned DNA, and the polypeptides apparently share a common amino acid sequence because both react with antiserum raised against a purified sog primase. Efficient synthesis of these polypeptides requires a promoter outside the 8.1 kb fragment, and since amber mutations prevented synthesis of the larger but not the smaller molecule, they are inferred to be separate translation products (10,42).

An important functional difference between the 2 polypeptides specified by sog is that only the 240 kd polypeptide has DNA primase activity. This activity requires a domain of between 36 and 75 kd at the N-terminal region of this large polypeptide, as estimated from sizes of proteins specified by deletion and amber mutants (10). Such a conclusion is consistent with the purification of an 87 kd

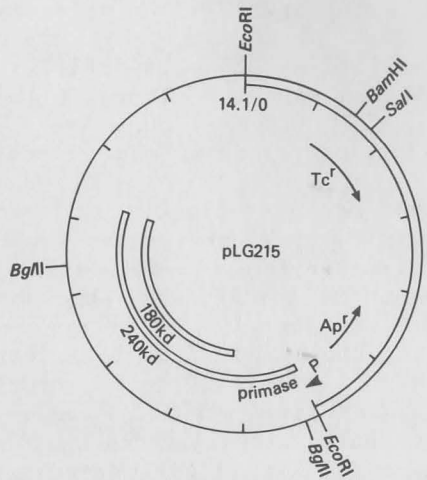


Fig. 1. Outline map of pLG215. The approximate locations of determinants of the 240 and 180 kd polypeptides are indicated, as deduced from the effect of deletion mutations that extend from the BamHI site without removing SalI. Efficient synthesis of both polypeptides requires transcription from the cat promoter of the vector (arrowhead labeled "P"). Further details are given elsewhere (10,42).

truncated polypeptide as a DNA primase with slightly greater specific activity than normal 240 kd sog product in the phage fd DNA assay system (42).

Overlapping genes with in-phase coding sequences have been detected in other genomes including those of phage λ and E. coli (36,37). The possible advantage of this type of gene arrangement is to allow formation of multifunctional protein complexes; regions common to both polypeptides may interact and carry out one function, while the domain that is unique to the larger molecule would participate in a separate reaction. As described in the next section, the primase moiety of the larger sog polypeptide acts to generate primers for conjugative DNA metabolism of $IncI_1$ plasmids but the role of the rest of this protein and the related 180 kd polypeptide is unknown. Elucidation will presumably require isolation and characterization of appropriate $IncI_1$ plasmid mutants.

The organization of the R16 pri gene has yet to be described. R16 belongs to the "I_1 complex" that includes plasmids of the I_1, I_1+B, B, and K incompatibility groups (12). These plasmids show significant DNA homologies and determine similar, thin, flexible pili that are serologically related (see Ref. 12 for a summary). Clearly members of the I_1 complex are heterogenous with regard to

primase genes since $IncI_1$ and R16 DNA primases are antigenically distinct. A survey (Ref. 15; Tab. 1) of a number of these plasmids showed that R64, R144, R621a, R864a, and RIP72 all contain nucleotide sequences homologous with the cloned ColIb-P9 sog gene. However, TP125, R724, pLG101 (IncB), R805, and R387 showed sequence homology with the R16 pri determinant but not with sog.

It is likely that many genetically different conjugation systems exist among plasmids of gram-negative bacteria (44) and since sog is apparently a transfer gene, it is possible that plasmids containing sog-like sequences specify the same system. If so, such plasmids should encode similar pili, which are required for cellular interactions in conjugation, and mutually interchangeable proteins for DNA transfer itself. Members of the I_1 complex can be arranged into different sets using pilus-specific phages Iα and PR64FS; the former adsorbs to the shafts of pili encoded by $IncI_1$ plasmids, R144 and R864a ($IncI_1$+B), and R621a (IncB), whereas PR64FS has a wider range since it also recognizes the tips of pili specified by R805a ($IncI_1$+B), and R387 (IncK) (Ref. 12; D.E. Bradley, pers. comm.). Hence, plasmids determining sensitivity to phage Iα possess sog-like sequences but the converse does not hold, as shown by RIP72.

At least 2 different DNA transfer mechanisms are recognizable among I_1 complex plasmids through the ability of representative plasmids to mobilize a small recombinant plasmid (pLG2009) carrying the origin of transfer (oriT) of ColIb-P9. Table 1 shows that $IncI_1$ plasmids, R144, R864a, and R621a mobilized pLG2009 with high efficiency. Thus these plasmids determine functionally similar DNA

Tab. 1. Mobilization of a recombinant plasmid carrying oriT of ColIb-P9 by conjugative plasmids of the I_1 complex.

Conjugative plasmid	Inc group	Primase gene type	Mobilization of pLG2009 [a]
ColIb-P9*drd-1* [b]	I_1	*sog*	82
R64*drd-11*	I_1	*sog*	513
R144*drd-3*	I_1+B	*sog*	50
R864a*drd-1*	I_1+B	*sog*	128
R621a	B	*sog*	231
RIP72	B	*sog*	0.12
TP125	B	*pri*	0.16
R724	B	*pri*	0.24
R805a	I_1+B	*pri*	0.078
R387	K	*pri*	2.5

[a] pLG2009 is a derivative of the nonmobilizable vector plasmid pED825 (2.2 kb; Ref. 17) containing *oriT* of ColIb-P9*drd-1* on a 0.8 kb fragment. Values show yield of pLG2009-containing transconjugants expressed as a percentage of transconjugants harboring the conjugative plasmid. Donor strains were mutant at *recA*.

[b] pLG221 (ColIb-P9*drd-1 cib*::Tn*5*) was used to allow selection for kanamycin-resistance.

transfer systems; they also contain sog-like sequences and comprise the set determining sensitivity to the RNA phage Iα. The other plasmids in Tab. 1 mobilized pLG2009 with frequencies of at least 30-fold lower than ColIb-P9, indicating that they specify DNA transfer mechanisms differing from that of the $IncI_1$ plasmid. With the exception of RIP72, this second group contains sequences homologous with the R16 pri gene. Thus, this survey indicates that plasmids with sog-like sequences specify the same or closely related conjugation systems, but the association is not absolute.

The RP4 pri gene (3.2 kb) is located between coordinates 40.3 and 43.5 in the recent SphI map of the plasmid (27), and the locus specifies 2 antigenically related polypeptides of 118 and 80 kd. The putative promoter is about 5 kb upstream of the beginning of the gene. Results of deletion mapping, together with immunological cross-reaction of proteins, indicates that the 2 polypeptides are separate in-phase translation products. Unlike sog products, both the 118 and 80 kd pri polypeptides possess DNA primase activity and this is located in a region towards the C-terminus of the products (26,28).

ROLE OF $IncI_1$ PLASMID PRIMASE IN CONJUGATION

Sog^- Mutants and Conjugative DNA Synthesis

We recently reported the isolation of a Sog^- $IncI_1$ plasmid called pLG250 and its use to identify the physiological role of the DNA primase (11). This mutant is a derivative of pLG221, which is ColIb-P9drd-1 conveniently marked by the presence of Tn5 in the colicin Ib gene, and it contains a hydroxylamine-induced mutation(s) that eliminates primase activity without affecting the apparent molecular masses of the sog polypeptides. Plasmid pLG250 was stably maintained in E. coli, implying that sog primase is not required for vegetative replication of the plasmid. Presumably the primers necessary for this process are generated by bacterial primase. However, the mutant was approximately 80% deficient in generating transconjugants in E. coli and Salmonella typhimurium matings, suggesting that the enzyme functions during conjugative synthesis of plasmid DNA. Consistent with this are the observations that sog is coordinately expressed with transfer genes, as judged by the effect of drd mutations in derepressing expression of these genes (41), and that sog activity and transfer proficiency were reduced by insertion of Tn5 in the E-2 fragment of ColIb-P9drd-1 (unpub. data).

During conjugation, a specific $IncI_1$ plasmid strand is transferred to the recipient cell (40) where the complementary strand is synthesized by DNA polymerase III holoenzyme in a process independent of RNA polymerase and E. coli primase (9,43). Involvement of sog primase in this process was investigated at the molecular level by measuring conjugative DNA synthesis in recipient cells (RCDS).

This was detected by measuring [^{14}C]thymine incorporation when thymidine kinase-deficient (tdk) donors were mated with thymine-requiring recipients under conditions that prevented the latter cells from replicating the resident DNA. This inhibition was achieved by exposing the cells to a large dose of ultraviolet light before mating, thereby inducing sufficient pyrimidine dimers to render the DNA unusable as a template for DNA synthesis (11).

Figure 2 shows the amount of RCDS detected when pLG221 (Sog^+) and pLG250 (Sog^-) donors were mated with irradiated dnaG3 recipients that were treated with rifampin to inhibit transcription of transferred DNA and thus to prevent synthesis of sog primase in transconjugant cells. Clearly RCDS in this mating system is dependent on plasmid primase which, in view of the rifampin treatment, is presumably supplied by the donor cell. The primary defect in the pLG250-mediated mating is not failure of DNA transfer itself, because the deficiency of RCDS was alleviated partially when the recipient cells harbored the pBR325-sog^+ recombinant pLG215 (Tab. 2) and thus contained plasmid primase before mating. We therefore infer that sog

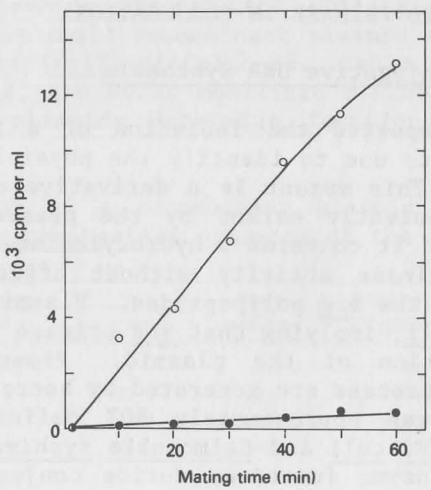


Fig. 2. Conjugative DNA synthesis in UV-irradiated, rifampin-treated dnaG3 recipients. Donor strain BW96 (dna^+ tdk rpoB) containing pLG221 (o) or the Sog^- derivative pLG250 (•) was mated at 41°C with recipient strain BW86 (dnaG3 uvrB thyA), UV-irradiated with 400 J/m^2 before use. Mating was in the presence of rifampin (100 µg/ml) and [^{14}C]thymine (63 mCi/mmol); TCA-precipitable radioactivity was determined at the times indicated. Further details are given elsewhere (11). Data have been corrected by subtraction of counts incorporated by unmated bacteria (maximum of 400 cpm per ml).

Tab. 2. Contribution of dnaG and sog primases to conjugative DNA synthesis in recipient cells.

Strains		Relative amount
Donor	Recipient	of RCDS
dna$^+$(pLG221)	*dnaG3*	1.0
dna$^+$(pLG250)	*dnaG3*	0.05
dna$^+$(pLG250)	*dna*$^+$	0.15
dna$^+$(pLG250)	*dnaG3*(pLG215)	0.37
dna$^+$(pLG250,pLG215)	*dnaG3*	0.64

BW96 was donor strain. BW86 and the homogenic *dna*$^+$ strain BW89 were recipients. pLG221 (Sog^+) and pLG250 (Sog^-) are IncIα plasmids while pLG215 is a pBR325-*sog*$^+$ recombinant. Matings were as for Fig. 2. Values are expressed as the amount of RCDS detected at 60 min relative to that when BW96(pLG221) was mated with BW86 (line 1).

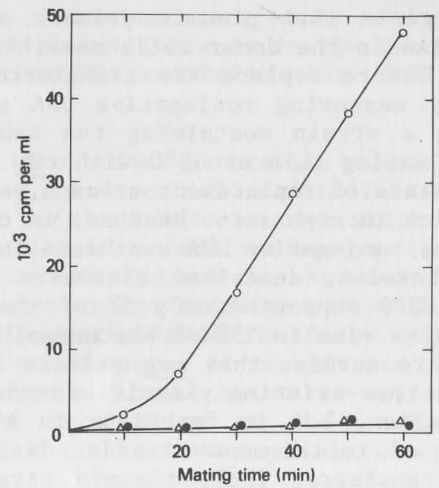


Fig. 3. Conjugative DNA synthesis in unirradiated, rifampin-treated dnaG3 recipients. Donor strain BW96 containing pLG221 (o) or pLG250 (•) was mated with BW86 recipients at 41°C. Counts incorporated when BW96(pLG221) was mated with BW86(pLG252) are also shown (Δ). pLG252 is a pBR325-eex^+ recombinant that excludes entry of pLG221. Other details are as for Fig. 2.

ISBN 0-306-41901-7